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Gil Mor

Immunology of Pregnancy

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To my wife Anette for her unconditional love and support

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PREFACE

Immunology of Implantation:
An Introduction

Gil Mor

Pregnancy Represents an Allograft

Cases of recurrent abortions, preeclampsia or babies born with hemolytic diseases of the newborn still puzzle us with the of the question "Why did your mother reject you?" Although, after looking at the complexity of the maternal-fetal immune interaction and the cases of successful pregnancies, with surprise and admiration the question now becomes: "Why didn't your mother reject you?"

Medawar, in the early 1950s, recognized for the first time the unique immunology of the maternal-fetal interface and its potential relevance for transplantation. In his original work, he described the "fetal allograft analogy" where the fetus is viewed as a semiallogeneic conceptus that evaded rejection. The approaches over the next 50 years have followed the methodology and development of transplantation immunity or more recently tumor immunity, unveiling new hypotheses and redefining old concepts.

The objective of this book is to review some of the significant events involved in human implantation related to the interaction between the maternal immune system and the fetus. The volume focuses on the main aspects of reproductive immunology, both from basic sciences and clinical points of view. Although there are still gaps in our knowledge, the advances accomplished in the last five years have proved the importance of understanding the role of the immune system during pregnancy. This not only represents a fascinating field for research, but it has the potential for new areas of treatment and diagnosis.

Defining Immunology of Pregnancy

Colbern and Main in 1991 redefined the conceptual framework of reproductive immunology as maternal-placental tolerance instead of maternal-fetal tolerance, focusing the interaction of the maternal immune system on the placenta and not on the fetus.[1] The embryo in early development divides into two groups of cells, an internal, the inner cell mass, which give rise to the embryo and an external layer, the embryonic trophoblast that becomes trophoblast cells and later the placenta. The cells from the placenta are the only part of the fetus to interact directly with the mother's uterine cells, and therefore the maternal immune system, and are able to evade immune rejection. The fetus itself has no direct contact with maternal cells. Moreover, the fetus per se is known to express paternal major histocompatibility complex (MHC) antigens and is rejected as allograft if removed from its cocoon of trophoblast and transplanted to the thigh muscle or kidney capsule of the mother.

This book we will focus on the interaction between trophoblast cells and the maternal immune system.

Immunology of Pregnancy, edited by Gil Mor.

General Concepts of Immunology

Types of Immune Response

The immune system eliminates foreign material in two ways: natural/innate immunity and adaptive immunity. Natural immunity produces a relatively unsophisticated response that prevents access of pathogens to the body. This is a primitive evolutionary response that occurs without the need of prior exposure to similar pathogens. For example, macrophages and granulocytes engulf invading microorganisms at the site of entry. Adaptive immunity is an additional, more sophisticated response found in higher forms such as humans. Cells of the innate immune system process phagocytosed foreign material and present its antigens to cells of the adaptive immunity for possible reactions. This immune response is highly specific and normally is potentiated by repeated antigenic encounters.

Adaptive immunity consists of two types of immune responses: humoral immunity, in which antibodies are produced and, cellular immunity, which involves cell lysis by specialized lymphocytes (cytolytic T cells). Adaptive immunity is characterized by an anamnestic response that enables the immune cells to 'remember' the foreign antigenic encounter and react to further exposures to the same antigen faster and more vigorously and by the use of cytokines for communication and regulation of the innate immune response.

Cytokines: Th-1 and Th-2 Type

Immune cells mediate their effects by releasing cytokines and thus establishing particular microenvironments. T helper lymphocytes (Th) that originate from the thymus play a major role in creating a specific microenvironment for a particular organ or tissue. Following an immune challenge, immune cells produce cytokine, the type of which determines their differentiation into T helper-1 (Th-1) or T-helper 2 (Th-2) lymphocytes. For example, Th-1 lymphocytes secrete interleukin-2 (IL-2) and interferon-γ (INF-γ) setting the basis for a pro-inflammatory environment. Conversely, the Th-2 lymphocytes secrete cytokines such as IL-4 and IL-10 which are predominately involved in antibody production following an antigenic challenge. The actions of the two types of lymphocytes are closely intertwined, both acting in concert and responding to counter regulatory effects of their cytokines. For example Th1 cytokines produce pro-inflammatory cytokine that while acting to reinforce the cytoytic immune response, also down-regulate the production of Th-2 type cytokines.[2]

Each of the different components of the immune system interacts, at different stages and circumstances, with the trophoblast. Our objective is to understand the type of interaction and its role in the support of a normal pregnancy.

In the following pages I will summarize some of the main hypotheses proposed to explain the trophoblast-maternal interaction.

Maternal Immune Response to the Trophoblast

The Pregnant Uterus as an Immune Privileged Site

Implantation is the process by which the blastocyst becomes intimately connected with the maternal endometrium/decidua. During this period, the semi-allogenic fetus is in direct contact with the maternal uterine and blood-borne cells; however, as I pointed above, fetal rejection by the maternal immune system, in the majority of the cases, is prevented by mechanism(s) yet undefined. A number of mechanisms have been proposed to account for the immune-privileged state of the decidua. The different hypothesis can be summarized in five main ideas: (i) a mechanical barrier effect of the trophoblast, (ii) suppression of the maternal immune system during pregnancy, (iii) the absence of MHC class I molecules in the trophoblast, (iv) cytokine shift, and more recently (v) local immune suppression mediated by the Fas/FasL system. I will discuss some of these hypotheses in brief and refer to the chapter where it is discussed in detail.

Mechanical Barrier

The concept of mechanical barrier was proposed to explain the lack of immune response in organs such as the brain, cornea, testicles and kidneys. We refer to these tissues as immune privileged sites where an immune response represents a dangerous condition for the tissue. Immune privilege sites are also organs or tissues of the body which, when grafted to conventional (nonprivileged) body sites, experience extended or indefinite survival. Whereas foreign grafts placed at nonprivileged sites are rejected promptly. The pregnant uterus is an example of an immune privilege site.

The first reasonable explanation of immune privilege was proposed by Peter Medawar in the late 1940s.[3] Medawar proposed that organs such as the anterior chamber of the eye and the brain resided behind blood:tissue barriers. The existence of a mechanical barrier, (in the brain the blood brain barrier [BBB]), prevents the movement of immune cells in and out of the tissue.[4] This barrier created a state of "immunologic ignorance" in which antigens within were never detected by the immune system without. The pregnant uterus was proposed to have a mechanical barrier formed by the trophoblast and the decidua, which prevented the movement of activated T cells from the periphery to the implantation site. Similarly, this barrier would isolate the fetus and prevent the escape of fetal cells to the maternal circulation.

Challenging the mechanical barrier effect theory are studies showing that the trophoblast-decidual interface is less inert or impermeable than first envisioned. Evidence for traffic in both directions across the maternal-fetus interface includes the migration of maternal cells into the fetus and the presence of fetal cells in the maternal circulation.

This is the case of almost all the immune privilege tissues, including the brain's BBB. Conclusive evidence has shown that immune cells circulate through all parts of the brain,[5] indicating that immune cells are not deterred by mechanical barriers.

The studies described by Adams and Lee Nelson in this book further demonstrate the bi-directional traffic across the maternal-fetal interface.

Systemic Immune Suppression

The second theory postulates the existence of nonspecific immune suppression during pregnancy. Numerous factors produced and isolated from the maternal placenta interface or from the serum have been associated with immunosuppressive activity. Some studies have suggested that human placental lactogen, human placental protein 14, and pregnancy associated plasma protein-A may have immune-depressant activity on lymphocytes. Soluble suppressor activity has also been identified in supernatants and cytosol fractions from placental explants and uterine secretions (for review see ref. 6). Although all these studies have shown an immunologic effect, it is important to keep in mind that many of these factors have only been partially purified and their action has been tested using in vitro assays for lymphocytes or NK cell activity. These assays are very sensitive to impurities, and upon further purification many of these factors have lost their "immunosuppressive" effects.

Progesterone has been suggested to have immunosuppressive effects.[7] Progesterone, in vitro, was described to be highly suppressive of mitogen activation and cytotoxic T-cell generation.[8] Similarly, progesterone was shown to blunt an inflammatory response in an in vivo rat model. Other studies have shown that progesterone inhibits cytotoxic and natural killer cell activity as well as prostaglandin F 2α synthesis. It has also been shown that progesterone activates regulatory T cells of a suppressor phenotype by induction of a 34 kDa protein from lymphocytes.[8-10]

The concept of systemic immunosuppressive has been studied by numerous investigators and for many years became an accepted explanation. Indeed, as described above, a wide array of materials in human serum have been found to have profound in vitro immunosuppressive activity. However, from an evolutionary point of view, it is difficult to conceive pregnancy as a stage of immune suppression. In cultures where a pregnant woman is exposed to poor sanitary conditions, a suppressed immune system would make fetus survival impossible. Furthermore, there are recent studies clearly demonstrating that maternal antiviral immunity is not affected

by pregnancy. The obvious observation that HIV+ pregnant women do not suffer from AIDS-like disease argues against the existence of such nonspecific immune suppression.

Lack of Expression of HLA Antigens

The third, more recently postulated theory is based on the fact that polymorphic class I and II molecules have not been detected on the trophoblast.[11] Dr. Schust's chapter discusses the subject in greater detail. Major histocompatibility complex (MHC) class I antigens are expressed on the surface of most nucleated cells and serve as important recognition molecules concerned with vertebrate immune responses. In humans, these antigens are also known as human leukocyte antigens (HLA). HLA class I genes are located on the same chromosomal region (6p.21.3). They have been subdivided into two groups, namely the HLA class Ia and the HLA class Ib genes, according to their polymorphism, tissue distribution and functions. HLA-A, -B and -C class Ia genes exhibit a very high level of polymorphism, are almost ubiquitously expressed among somatic tissue and their immunological functions are well established: they modulate antiviral and antitumoral immune responses through their interaction with T and NK cell receptors. In contrast, HLA-E, F and G class Ib genes are characterized by their limited polymorphism and their restricted tissue distribution. Their roles are still poorly understood. The human placenta does not express HLA-A and HLA-B class I antigens but expresses HLA-G and HLA-C molecules.[12] Where are those genes expressed? Dr. Schust's review discusses this question.

Cytokine Shift

The proliferation, invasion and differentiation of trophoblast cells during implantation is a tightly controlled process coordinated by a system of intercellular signals mediated by cytokines, growth factors and hormones.[13,14] An extensive array of cytokines is produced at the trophoblast-maternal interface that contributes to the well being of the feto-placental unit. Furthermore, these cytokines to a great extent regulate maternal immune responses, which play an important role for a successful pregnancy outcome.

It is now recognized that cyokines have extremely diverse biological effects which may involve cell growth, differentiation and function. Their role in regulating human placenta development and implantation has been much discussed in recent years.[15] The field of cytokines and implantation could be divided in two aspects, one is their role as regulators of the immune response and second as factors controlling trophoblast cell growth and implantation. This subject is extensively reviewed by Dr. Shigeru Saito, Dr. Surendra Sharma, Dr. Jan-S. Krüssel and Dr. Aydin Arici.

Local Immune Suppression

The last main hypothesis that we will discuss in this review is the "specific antipaternal suppressor/regulatory mechanism" observed during pregnancy. The first set of observations pointing towards the importance of local immune regulation was from Rossant and colleagues. Their observations were done using the *Mus musculus*:*Mus caroli* system (for more details in the model see ref. 16). They have shown that the transfer of *M. musculus* eggs into *M. caroli* is always successful; in contrast, there is almost a constant time schedule for failure of *M. caroli* embryos in the *M. musculus* uterus. In such a case, cotransferred adjacent *M. musculus* embryos do survive, whereas all the *M. caroli* embryos die from almost the same program. A strong immune infiltrate consisting of CTL and NK cells is observed around day 9.5. By day 13, the embryos are all completely reabsorbed.[17] It was later shown that *M. caroli* embryos can survive until delivery, provided that M. *musculus* placenta was used.[18,19] These results suggested that an important part of the placenta in *M. caroli* origin was responsible for provoking death and resorbtion of *M. musculus* embryos.[19]

This model was the first to describe these immunologically-mediated abortions and revealed the "immunological" role of the placenta. Furthermore, we consider that one of the

great merits of this model was to bring to focus the importance of local immunoregulatory events.

More recently, evidence exists for specific immune suppression directed towards the paternally encoded histocompatibilty antigens. Here, the maternal T cells that recognize paternal antigens on the trophoblast are selectively abrogated. The role of decidual T cells during pregnancy is discussed by Dr. Lucia Mincheva-Nilsson.

The Role of the Innate Immune System in Pregnancy

During normal pregnancy, several of the cellular components of the innate immune system are found at the site of implantation. Furthermore, from the first trimester onwards, circulating monocytes, granulocytes and NK cells increase in number and acquire an activated phenotype. This evidence suggests that the innate immune system is not indifferent to the fetus and may have a role not only in host protection to infections, but also as important players in the feto-maternal immune adjustment.

Vikki Abrahams, Ulrike Kaemmerer, Ali Ashkar and I discuss the possible roles of cells of the innate immune system during pregnancy.

Furthermore, Dr. Abrahams' chapter presents evidence supporting the hypothesis that the trophoblast can function as an immune cell, capable of recognizing and responding to bacterial antigens.

Apoptosis and Implantation

During implantation, the uterine endometrium undergoes morphological and physiological changes to accommodate the embryo. This process of accommodation implies that the embryo has to degrade the endometrial extracellular matrix (ECM) to invade the uterus in species with hemochorial placentation. Apoptosis has been observed in endometrial epithelial cells at the embryo implantation site, and it is believed to be due to loss of contact with ECM. Those apoptotic cells are removed either by throphoblast or by maternal macrophages.

Apoptosis marks unwanted cells with "eat me" signals that direct recognition, engulfment and degradation by phagocytes.[20] This clearance process, far from being the end, represents an active and coordinated event, which will send specific signals to the remaining cells either for survival or death.[21] If the wrong message is sent by macrophages to the wrong cell type, it may have profound consequences for the normal physiology of the tissue.

Dr. Shawn Chavez discusses in detail the regulation of apoptosis in trophoblast cells.

Summary

Important reproductive events, including implantation, trophoblast invasion, placental development and immune protection are regulated by immune cells and their products (cytokines) produced at the maternal-fetal interface.

The maternal-fetal immune interaction is very complex, and it is difficult to perceive the whole process based on one mechanism of action. Clearly there are multiple mechanisms of peripheral and local tolerance induction during pregnancy that prevent fetal rejection while maintaining a strong and active immune surveillance against viral or bacterial infections, which may endanger the successful outcome and the survival of the species.

Some of these mechanisms are discussed in this book. In addition the chapters of Drs. Romero, Lockwood, Krüssel, Kwak-Kim and Richman present a clinical view of the role of the immune system in normal pregnancy and how its alterations may lead to complications of pregnancy.

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CHAPTER 1

Evolution of the Mammalian Reproductive Tract and Placentation

Susan Richman and Frederick Naftolin

Abstract

Phylogenetic analysis suggests that the internalization of reproduction and the development of hemochorial placentation have been accompanied by conservation of primitive genitourinary genes. The products include the renin-angiotensin system and the innate immune system. This explains what might otherwise be considered an ectopic presence of these systems in the mammalian reproductive tract and the interaction of the allograft embryo and maternal host.

Introduction

Evolution is a conservative process; it more often proceeds through utilization of previously neutral characters than depending upon de novo mutation and selection: novel applications generally arise via utilization of preexisting adaptive mechanisms. Classical evolutionary methodology uses the fossil record, in conjunction with observations of both extant species and ethnographic evidence from surviving societies.[4] For example, the length of human gestation and challenges of delivery such as cephalo-pelvic disproportion appear consequential to the assumption of an upright posture combined with cranial expansion. At the molecular level, this is accomplished by complex combinations of gene duplication, exon shuffling, and transposition. For example, the ancient glycoprotein hormone chorionic gonadotropin (CG) acts as a signal to maternal physiology to begin a series of adaptations to pregnancy. The mammalian gene for CG's beta subunit arose by duplication of the LH beta subunit gene approximately 94 million years ago from the common ancestor of both eutherian mammals and anthropoid primates. During that time span, the gene duplication was apparently followed by a frameshift mutation in the third exon.[1] The major difference in CG gene function from its ancestral LH is in gene expression variants, composition and length of coding region. The translated products differ in the number of sugar chains attached, slowing the clearance of CG molecules from the maternal bloodstream to 12 hours, from 30 minutes in the case of LH.[2] Analogous changes occurring in the structure and function of the excretory apparatus have led to the development of the mammalian reproductive tract and placentation.[3]

Mammalian Reproduction

The development of sexual reproduction fostered genetic variability, which has hastened the pace of evolution. The transition from external to internal fertilization shielded reproduction from a hazardous external environment (predators, toxic chemicals, adverse temperature and pH), which has resulted in the requirement for fewer gametes per successful conception.

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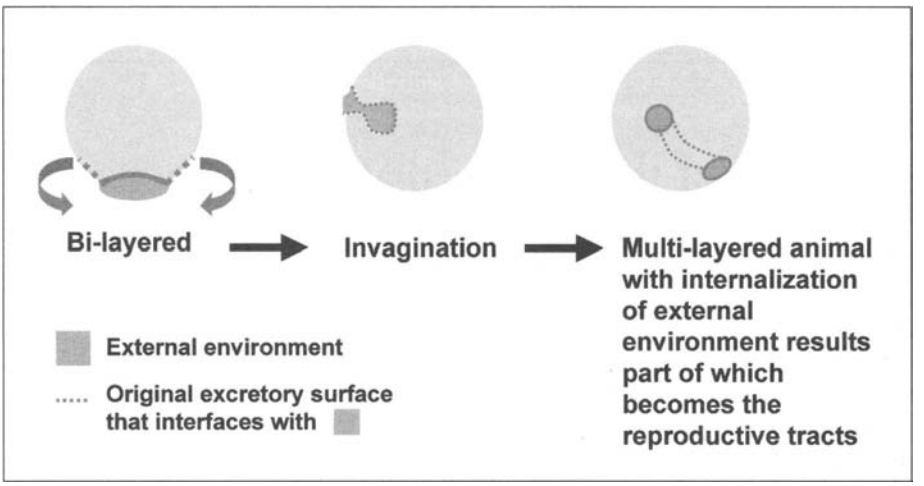

Figure 1. Development of sexual reproduction: adaptation from external to internal reproduction.

Internal fertilization has been accomplished by the enfolding of excretory and reproductive function. This adaptation accompanied the development of nonaquatic, terrestrial life forms, including mammals (Fig. 1).

The higher proportion of live-born young resulting from this system requires a higher investment per oocyte, but furnishes greater overall reproductive success, gene transmission and speciation. In humans, the allocation of resources that might have been devoted simply to generation of innumerable eggs for external fertilization has been replaced by the cyclic modification of the reproductive organs, sexual activity, placentation, gestation, parturition and lactation.[5] All of this developed in the remnants of the ancient excretory tract, with the preservation of many of its mechanisms for interacting with an aquatic external environment.

Secondary Use of Immune Mechanisms for Reproduction

Molecular features of invertebrate immune systems such as the immune effector cells have been retained in mammals. Three genes found in echinoderms encode highly conserved transcription factors; NF-κB, GATA-2/3, and Runt-1, which are rapidly upregulated in response to bacterial challenges. SRCR family genes structurally resemble the mammalian macrophage scavenger receptors.[6] Vertebrates added to this successful strategy by:

1. Internalizing mucosal surfaces and increasing their complexity to form the reproductive tracts—internalized but still aquatic environment.
2. Retaining control over the entirety of embryo development within the female reproductive tract, allowing the young to be born at more advanced stages of development. This, in combination with maternal supervision and protection, facilitates evasion from predators.

Creating this microenvironment for gametogenesis, fertilization and implantation, was accomplished by the aforementioned "internalizing" of the extracorporeal space within the modern reproductive tract. In the process, ancient nonreproductive systems such as the macrophage-cytokine system (innate or nonspecific immunity), which had evolved to interface the genital precursor with the external environment and invading organisms, were modified to accommodate the embryo. Mucosal immunity at body surfaces via TCR (T cell antigen receptor) γδ lymphocytes emerged earlier in evolution than TCR αβ, perhaps due to primitive digestive tract exposure to injury and infection in early jawed vertebrates.[7] The generation of T cells also occurs in gut associated lymphoid tissue, which was the early adaptive immune

system, while the thymus evolved later, and its ontogeny is from pharyngeal pouch endoderm. In humans, the third pouch develops into the thymus, while the second develops into the palatine tonsil. The thymus also utilizes evolutionarily conserved immune-neuroendocrine effectors, as its mesenchyme develops from neural crest cells. T and B cells, MHC and antibody production constitute the adaptive or specific portion of the immune system.[8]

Signals from the embryo-host interaction relay the presence of an allograft to the maternal host, triggering the deployment of processes originally designed to protect against microbial or environmental challenges.

A later chapter will describe how hormonal regulation of immunocytes prevents rejection of the allograph embryo; however, the evolutionary relationship between the endometrium and the embryo is a derivative function of the reproductive tract development.

The Role of the Endometrial Cycle

It is conventional to consider the ovarian and endometrial cycles as the fundamental processes involved in reproductive biology. However, the primary biologic goal is **reproduction**, and menstruation is merely the avenue of reestablishing reproductive competence. In an evolutionary sense, each complete menstrual cycle signals a lost opportunity to perpetuate the germ line.[9]

The superficial endometrium (functionalis) is the nexus of fetal signaling and the adhesion/implantation mechanism.[10] In higher primates, this portion of the endometrium will be shed periodically. This occurs in the absence of signals (hCG, etc.) from the conceptus that drive the corpus luteum's cells to secrete the estrogen and progesterone that decidualize the endometrium and maintain the embryo until its placenta is able to function independently. The complete mechanism of menstruation (shedding of the functionalis) following ovulation remains unsettled; it appears that this process is triggered by the withdrawal of ovarian steroids from the expiring corpus luteum that upregulate production of PGF2α.[11] VEGF secreted by the endometrial stromal and epithelial cells plays a role in the remodeling and regeneration from the basalis layer that follows in the subsequent cycle, providing another opportunity to achieve pregnancy.[12]

The unique individual that is at the blastocyst stage will invade the receptive endometrium and become essentially an allograft. This occurs in two steps: adhesion followed by implantation. The yolk sac-placenta provides nourishment until the definitive placenta develops. The maternal host's reaction to invasion by the embryo includes ancestral innate immune reactions to foreign proteins, modulated by estrogen, progesterone, and other signals from the maternal gonad and/or embryo. At this point, immune function is primarily a TH1 response.[13]

The human placenta is uniquely aggressive, and capable of invading through the endometrium to the myometrium and beyond, as in the case of placenta accreta/percreta. It is not yet clear what role this characteristic plays in. Balancing the need for minimally encumbered respiratory exchange, against the danger of overzealous invasion leading to maternal exsanguinations or other complications. While the villous cytotrophoblasts are extraordinarily efficient for this respiratory and nutrient exchange, the invasive extravillous cytotrophoblast must be limited to invading only the decidua and superficial myometrium. Without this control, the placenta could implant on muscle that would not provide proper nourishment to the conception and the mother would risk exsanguination from her large pelvic vessels. Potential controlling autocrine/paracrine mechanisms include glycoproteins, cytokines, and growth factors.[14] The proliferative, invasive and migratory activity of the villous cells declines with increasing gestational age, but it has not been established whether this is due to intrinsic cell programming or extrinsic decidual factors.[15]

Immunoregulatory mechanisms are increasingly seen to be key regulators of this invasive behavior. In vitro models of the maternal fetal interface involve co–culture of trophoblast and decidual cell lines on collagen gel matices. Decidual TBF-B and dermatan sulfate proteoglycan

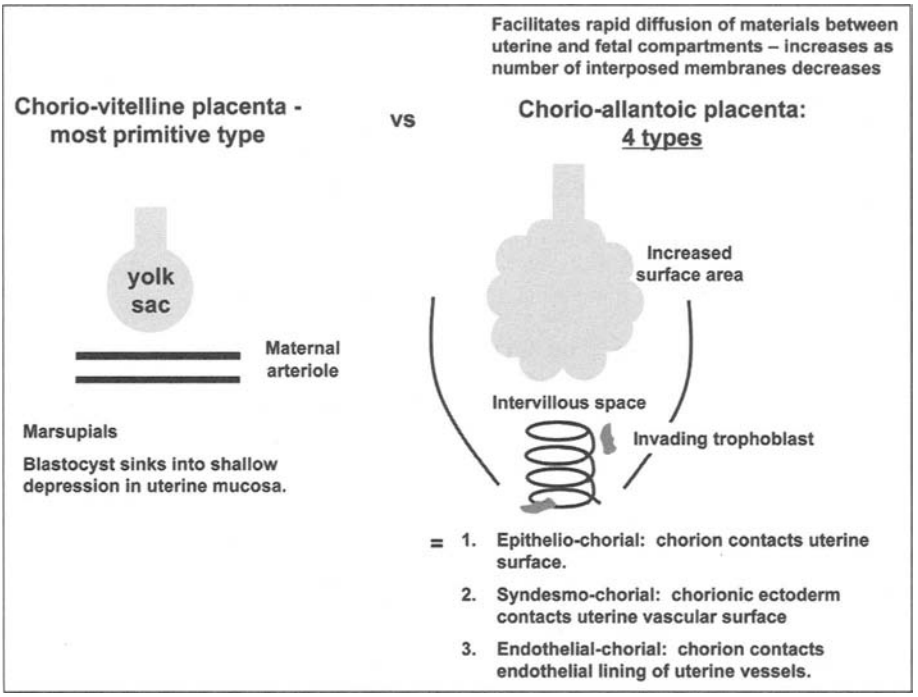

Figure 2. Adaptational changes on placentation.

II have been shown to prevent overinvasion when activated by trophoblast proteolytic enzymes such as MMP.[15] During placenta development, lymphocytes are excluded form the maternal-fetal interface, while monocytes and granulocytes gain access.[16]

Endometrial stromal cells and deciduas express insulin, IGF-1, and glucocorticoid receptor, peaking at days 4 and 5 of gestation.[17] This suggests a relationship between the regulation of invasion and the immunologic alterations in the progression of pregnancy, i.e., the barrier may be one and the same: the immune system.

Placentas and Placentation

The most primitive and presumably ancestral placentation is choriovitelline, formed by fusion of the yolk sac and chorion. Placental structural evolution proceeded towards the generation of a larger surface area, which facilitated metabolic exchange accompanying changes in more aggressive invasion of the maternal host. The production of growth factors, cytokines and hormones encourages increased blood flow and nutrient delivery to the feto-placental unit. Interspecies comparisons again demonstrate the recycling of existing pathways for functions common to other systems, such as the FGF signaling and branching morphogenesis utilized in ontogeny of both pulmonary alveoli and placental villi (Fig. 2).[18]

Study of placental structure in eutherian mammals suggests adaptive pressure for development of the hemochorial type of placenta over alternative epitheliochorial or synepitheliaochorial types. Hemochorial placentae are not found in any animal larger than the human or gorilla. This may be secondary to the potential drawback of such structure in the ready passage of fetal cells to the maternal organism and potential for oxidative stress.[19] Nucleotide sequence data suggests that haemochorial placentation evolved independently in each of the four mammalian

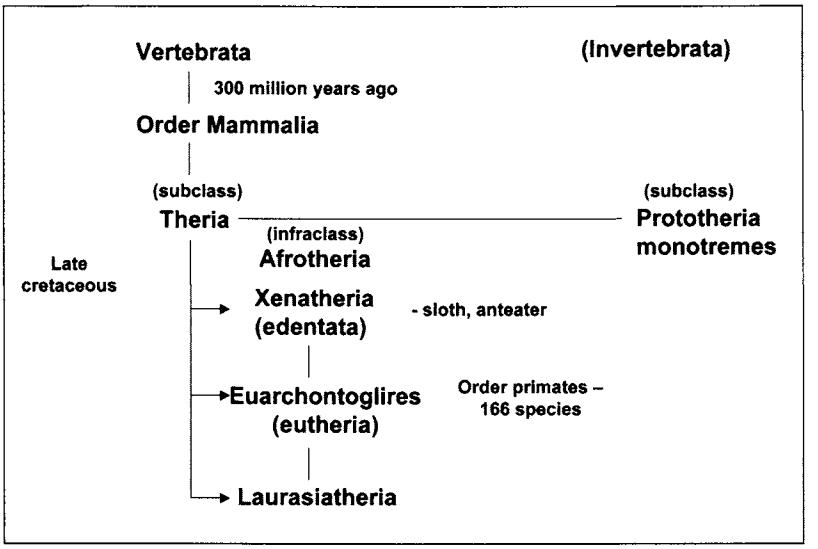

Figure 3. Species classification according to their placental development.

superorders, likely reflecting their separation by the newly emerging continental land masses 100 million years ago.[20] This is classical evolutionary adaptation as described by Darwin after his visit to the Galapagos Archipelago, in which side vent lava flows perform in the same manner as the spreading of the tectonic plates described above (Fig. 3).

The eutherian mammal branch is relatively recent, and there are few placental specific genes that appear to have arisen by gene duplications and deletions.[1] Primate- specific placental adaptations such as early implantation, deep and widespread invasion of trophoblast cells into and remodeling of maternal decidual vessels may be compensation for the biomechanical constraints imposed by bipedal posture.[21] However, it is associated with the most aggressively invasive placenta in nature, that of Homo sapiens.

Maternal-Fetal Immune Function

Placental Evolution

The ubiquitous challenge of balancing protection against invading foreign organisms with the necessity for the maternal immune system to tolerate the presence of a fetal endograft containing 50% nonself antigens is not unique to primates. Most maternal antibodies misdirected against the fetus are directed against paternally inherited MHC.

Mammalian TATA binding protein, used for promoter recognition during transcription by RNA polymerases in all eukaryotes, is another highly conserved molecule across species. Mice with an engineered version lacking 111 amino acids die in mid-gestation, despite normal transcription function of the enzyme complex, apparently due to structural placental defects that lead to maternal rejection type reactions. Embryonic rescue is possible by utilization of immuno-compromised mothers, suggesting that the TBP- N terminus disrupts a β2m-dependent process that the placenta uses to evade a maternal rejection response. This system is ubiquitous to all vertebrate species, and may have coevolved with the MHC system, as both are linked on chromosome 17.[22]

Placental Contribution and Graft Tolerance

Placental trophoblasts produce many immunosuppressive molecules, such as progesterone, matrix metalloproteinases, and complement inhibitors. Many species have solved this conundrum in a similar fashion, by minimizing the placental expression of major histocompatibilty complex genes. This occurs despite the gross structural differences. Vertebrates developed specific immunity, in contradistinction to the generalized defense systems such as mucus, cilia, enzymes, phagocytosis, and acute phase proteins. All vertebrates will reject tissue grafted from nonisogenic individuals of the same species, and exhibit the same degree of plasticity, necessary to keep pace with the short intergenerational intervals and frequency of mutations characteristic of invading pathogens. In parallel with the microorganisms, T and B cell intergenerational intervals are short, between 12 and 24 hours. B cell antibody receptors exhibit 10^8 specificities, while the corresponding number for T cells is 10^{15}. Positive and negative selection during thymic maturation reduces the risk of self reactivity in peripheral tissues. This is a central theme in the evolution of multicellular organisms, i.e., species success depends on the resolution of conflict between selection at the level of the multicellular entity versus that of the individual cell.[23]

In the case of mammals, fetuses are retained within the reproductive tract for longer periods of time, increasing the temporal challenge to the immune system.[24]

Of the MHC molecules, HLA-G has been most extensively studied, being expressed preferentially on extravillous trophoblasts at the maternal-fetal interface. It is one of the three nonclassical human MHC class I genes. Its expression on target cells protects them from natural killer (NK) cell-mediated lysis via inhibitory receptors 1 and 2. The CD94/NKG2-A receptor complex is most utilized by maternal decidual NK cells. HLA-G has been proposed as the ancestral MHC class I gene via sequence homologies.[25] These molecules do not present antigens and may send the above noted negative signals to maternal NK cells to avoid fetal rejection. HLA-DR antigen expression has also been sought on human first trimester trophoblasts without success.[26]

The low polymorphisms in HLA-G molecules worldwide in human populations and the lack of hypervariable regions at the peptide binding site argue for strong selection pressure for its perpetuation. Conserved intron 2 sequences in all primate species studied thus far suggest that this structure may have appeared as recently as 15 million years ago, when the orangutan diverged from the human lineage.

An alternative system that may be employed in the service of fetal immune tolerance is that of Fas/FasL. Activated T cells recognizing placental alloantigens express Fas, bind to the FasL expressed by the trophoblast, and undergo apoptosis.[27]

Comparative amino acid sequence analysis of IgE, G and G2 structure confirms the immunologic divergence of mammals from early reptilian species approximately 300 million years ago.[28] The mammalian immune system appeared approximately 100 million years ago at the time metatherian (marsupial) and eutherian placental lineages emerged (Fig. 3).

Summary

In summary, the evolution of the human reproductive tract and placentation demonstrates conservative retention of archetypal systems found in simpler species. These have been modified for the complexity of primate reproduction. These modifications include internalization of the excretory apparatus for use in reproduction (Fig. 4).

Accordingly, it is not surprising that the mechanisms involved in interactions between cells and tissues occupying the reproductive tract and the tract itself are the same as those used in interactions between the body and its (internalized) extracorporeal space.

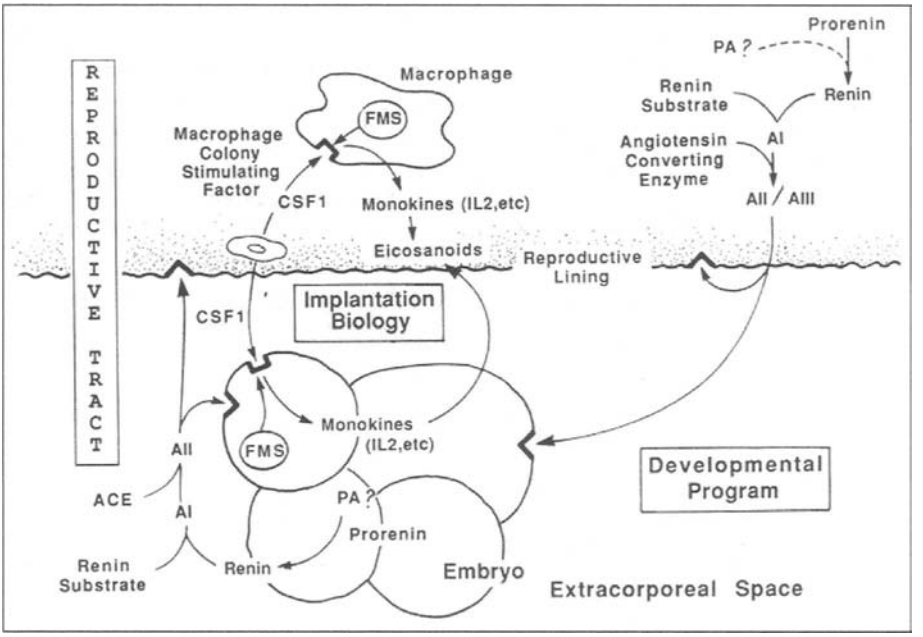

Figure 4. A summary of the relationship between the lining of the reproductive tract and the developing embryo. Note that the embryo, which is in the uterine cavity, is within the extracorporeal space that has been incorporated by the internalization of the reproductive process, see Figure 1. (Modified from Naftolin et al. Gynecological Endocrinology 1988; 2:265-273.)

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CHAPTER 2

Toll-Like Receptors and Pregnancy

Vikki M. Abrahams and Gil Mor

Abstract

The maternal-fetal interface represents an immunologically unique site that must promote tolerance to the allogenic fetus, whilst maintaining host defense against a diverse array of possible pathogens. Clinical studies have shown a strong association between certain pregnancy complications and intrauterine infections. Therefore, innate immune responses to microorganisms at the maternal-fetal interface may have a significant impact on the success of a pregnancy. There is growing evidence that trophoblast cells are able to recognize and respond to pathogens through the expression of Toll-like receptors, a system characteristic of innate immune cells. This review will discuss the role of Toll-like receptors at the maternal-fetal interface, the potential for trophoblast cells to function as components of the innate immune system and the impact TLR-mediated trophoblast responses may have on a pregnancy.

Introduction

During pregnancy there is a strong immunological presence at the maternal-fetal interface, particularly by cells of the innate immune system.[1] The role of the immune system at the maternal-fetal interface is thought to facilitate implantation and placental development, whilst promoting fetal tolerance.[1-3] However, a certain level of host defense at this site is also required. As a consequence, either an inefficient clearance of an infectious agent, or an overzealous immune response may have a significant impact on the pregnancy. Clinical studies have shown a strong association between certain pregnancy complications and intrauterine infections,[4,5] suggesting that the innate immune response can affect the outcome of a pregnancy. Preeclampsia and intrauterine growth restriction (IUGR) are both thought to be associated with infection[6-8] and a link between preterm labor and intrauterine infections is now well established. Indeed, infections have been reported as responsible for up to 40% of preterm labor cases.[9] Furthermore, 80% of preterm deliveries occurring at less than 30 weeks of gestation have evidence of infection,[10] suggesting that an intrauterine infection may occur early in pregnancy, preceding such pregnancy complications.[4] Infection, therefore, represents an important and frequent mechanism of disease, yet, the precise molecular mechanisms by which infection can affect a pregnancy remains undefined. While immune cells such as macrophages and NK cells are present the maternal-fetal interface,[1] they may not be the only cells able to respond to infectious agents. In addition to the classical immune cells, placental cells may also have the potential to function as a component of the innate immune system.[11] This review will discuss how trophoblast cells may respond to a pathogen through the system of evolutionary conserved proteins known as Toll-like receptors, and how such responses might impact a pregnancy.

Infections and the Innate Immune

The innate immune system represents the immunological first line of defense against invading pathogens through is its ability to distinguish between what is non-infectious self and

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infectious nonself.[12] One way in which the innate immune system achieves this is through an evolutionary conserved system of pattern recognition.[13] Cells of the innate immune system express a series of receptors known as pattern recognition receptors (PRR) which recognize and bind to highly conserved sequences known as pathogen-associated molecular patterns (PAMPs). Pathogen-associated molecular patterns are unique to, and expressed on, the surface of microorganisms. Examples of PAMPs include lipopolysaccharide (LPS), the major component of gram-negative bacterial outer membranes, and peptidoglycan, the major component of gram-positive bacterial cell walls.[12] The ligation of PRR by PAMPs results in an inflammatory response generated against the invading pathogen.[14] Furthermore, activation of TLR expressed by antigen presenting cells, such as dendritic cells, may facilitate the initiation of adaptive immune responses.[15] There are a number of different PRR including the mannose-binding receptor and the scavenger receptor,[16] however, this review will focus on the major family of PRR, the Toll-like receptors.

Toll-Like Receptors

Originally discovered in Drosophila, the *Toll* gene was found to be critical for dorso-ventricular polarization during embryonic development.[17,18] However, later studies revealed that *Toll* also have anti-fungal and anti-bacterial properties in the adult fly.[19,20] Subsequently, mammalian Toll was identified and to date, 11 *Toll* homologues have been identified and designated, Toll-like receptor (TLR) 1-11.[21,22] Ligation of TLR by microbial products results in an inflammatory immune response characterized by the production of cytokines and anti-microbial factors. Furthermore, through the regulation of co-stimulatory molecules, TLR may also facilitate the development of adaptive immune responses.[14]

Toll-like receptors are transmembrane proteins which have an extracellular domain containing leucine-rich repeat motifs. Each receptor differs in their ligand specificity. So while individually, TLR respond to limited ligands, collectively the family of TLR can respond to a wide range of proteins associated with bacteria, viruses, fungi and parasites (Fig. 1). TLR-4 was the first human Toll-like receptor to be identified[23] and was subsequently found to be the specific receptor for recognition of LPS.[24-26] Early studies showed that overexpression of constitutively active TLR-4 in monocytes resulted in the upregulation of pro-inflammatory cytokines and costimulatory molecules, suggesting that this receptor is involved in both innate and adaptive immune responses.[23] TLR-4 recognition of LPS is thought to be potentiated by additional molecules. Prior to the identification of human TLR, LPS responses where thought to be initiated through CD14 which recognizes the LPS/LPS binding protein (LBP) complex.[27] It is now thought that following the binding of the LPS/LBP complex to CD14, TLR4 becomes either indirectly or directly activated. Another protein that appears to enhance LPS responses is MD-2.[28-30]

Of all the Toll-like receptors identified, TLR-2 has the widest specificity. TLR-2 binds to gram-positive, gram-negative and mycobacterial associated lipoproteins, gram-positive peptidoglycan and lipoteichoic acid, as well as fungal zymosan.[31-36] Indeed, TLR-2 deficient mice are highly susceptible to *Staphylococcal aureus* infections[37] and are unable to respond to either peptidoglycan or lipoproteins.[38,39] TLR-2 recognition of some microbial products appears to be dependent upon the formation of heterodimers with either TLR-1 or TLR-6.[40,41] TLR-2/TLR-1 recognize bacterial triacylated lipoproteins,[42] while TLR-2/TLR-6 complexes recognize mycoplasmal diacylated lipoproteins.[40,43] TLR-3 binds to viral dsRNA, TLR-5 binds bacterial flagellin, TLR-8 recognizes ssRNA and TLR-9 binds bacterial CpG DNA.[21,22,44] The natural ligands for human TLR-7 and TLR-10 are, as yet undetermined.

Toll-Like Receptor Expression

As expected, TLR are widely expressed throughout the cells of the immune system, specifically those of the innate. Toll-like receptors can also be expressed by non-immune cells, particularly if such a cell can contribute to an inflammatory response, and most tissues express at least one TLR.[45] Toll-like receptor expression by mucosal systems is important for host defense

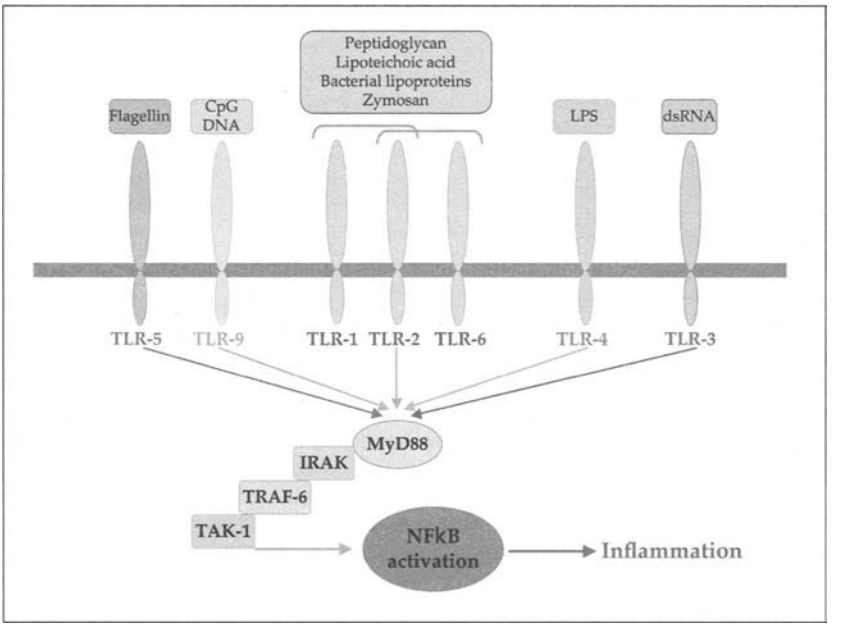

Figure 1. Toll-like receptor specificities and signaling. While all Toll-like receptors differ in their specificity, they can all signal to a common intracellular pathway through the signaling adapter protein, MyD88.

against pathogens.[46,47] While most studies have focused on the intestinal and respiratory tracts, there is growing evidence that the mucosal epithelium of the female reproductive tract (FRT) is also an important immunological site.[48,49] Indeed, Toll-like receptors are expressed by endometrial epithelium and the epithelial cells of the lower reproductive tract.[50,51] Furthermore, these cells are able to respond to microorganisms through these receptors.[50,52] These studies suggest that TLR play an important role in host defense within the normal cycling FRT, which raises the question of whether TLR also play a role in innate immune responses during pregnancy.

Toll-Like Receptors and Pregnancy

At present, little is known about the role of Toll-like receptors during pregnancy. Trophoblast cells from term placenta have been shown to express TLR-1-10 at the RNA level.[45,53] At the protein level TLR-2 and TLR-4 are expressed.[54] These findings suggest that trophoblast cells may interact with microorganisms present at the implantation site and initiate an immune response. The trophoblast may, therefore, function as an active member of the innate immune system, as was once proposed by Guleria and Pollard.[11] In our studies we have evaluated the expression Toll-like receptors by first trimester trophoblast cells. We have observed that in first trimester placental tissues, TLR-2 and TLR-4 are highly expressed. Interestingly, the trophoblast cell populations expressing these receptors are the villous cytotrophoblast and extravillous trophoblast cells. The syncytiotrophoblast cells do not express these TLR and this suggests that the placenta serves as a highly specialized functional barrier, protecting the developing fetus against infection. The lack of TLR expression by the outer trophoblast layer is analogous to studies of mucosal epithelial cells of the intestinal tract which have been shown to express TLR-5 and TLR-4 only on their basolateral side.[55] These cells will only respond to a bacterium that has invaded the basolateral compartment from the apical side. Since a pathogen is characterized as a microorganism that breaches certain physical barriers, these observations have helped to explain how an immune response can be mounted against pathogenic, but not commensal bacteria. Similarly, a

microorganism will only be a threat to the fetus if the TLR-negative syncytiotrophobast cell layer is breached and the pathogen has entered either the decidual or the placental villous compartments. Therefore, the placenta can distinguish between pathogenic and commensal microorganisms during pregnancy. Once an infection has gained access to the TLR positive trophoblast cells, a response may be mounted. As described below, the type of pathogen and, therefore, the specific receptor activated may have a significant impact on the type of response generated by the cells of the placenta.

Toll-Like Receptor Signaling

While extracellularly, each TLR is distinct in their specificity, all receptors signal through a common pathway (Fig. 1). Toll-like receptors have an intracellular domain which is highly homologous to the type-1 Interleukin-1 receptor (IL-1R) and is known as the Toll/IL-1R homology region (TIR).[56] Both TLR and the IL-1R recruit and interact with the adapter signaling protein, myeloid differentiation factor 88 (MyD88). MyD88 was first identified in macrophages and studies using MyD88-deficient mice have demonstrated the importance of this protein in TLR signaling.[57] MyD88 contains a TIR domain within its C-terminal and a death domain (DD) within its N-terminal.[58,59] Following ligation of a TLR by its ligand, MyD88 becomes associated with the intracellular domain of the receptor through a TIR-TIR interaction.[60-63] In turn, MyD88 through its DD recruits and activates the DD-containing serine/threonine kinase, IL-1R associated kinase (IRAK).[61] IRAK then dissociates from the receptor complex and becomes associated with TRAF-6.[64] Downstream activation of the NF-κB and MAP kinase signaling pathways occurs through activation of a kinase cascade which includes TAK-1 and IKK.[65,66]

Experiments using MyD88 deficient cells revealed that, NF-κB and JNK activation induced by TLR-3 and TLR-4, unlike other the TLR, was not completely abolished, but instead delayed.[67] These observations suggested that some Toll-like receptors could signal via MyD88-independent pathways (Fig. 2). In addition to MyD88, TLR-4 can associate with TRIF, which via its N-terminal can directly bind TRAF-6 and subsequently activates NF-κB.[63] Furthermore, both TLR-4 and TLR-3 in response to LPS and dsRNA respectively can stimulate the production of type I interferons (IFNα and IFNβ) and trigger the expression of IFN-inducible genes. This occurs as a result of TRIF, through TBK-1, also having the ability to activate the transcription factor, IFN regulatory factor (IRF-3/7).[63,68-71] These new findings highlight that while the family of Toll-like receptors share many features, some unique properties of individual receptors can have a significant impact on the immunological and functional outcome.

Toll-Like Receptor Signaling in Trophoblast Cells

The function of Toll-like receptors at the maternal-fetal interface is an area of research still in its infancy. At present we know that trophoblast cells from term placental explants can produce IL-6 and IL-8 following ligation of TLR-2 or TLR-4 by zymosan or LPS, respectively.[54] Treatment of term trophoblast cells with LPS has also been shown to induce the production of nitric oxide which has potent anti-microbial properties and MMP-2.[72] In addition, studies on first trimester trophoblast cells have shown that treatment with LPS induces the production of G-CSF and RANTES.[73] Recent work from our laboratory has focused on the function of Toll-like receptors during the first trimester of pregnancy. We have found that activation of TLR-4 by LPS triggers trophoblast cells to generate a classical TLR response, characterized by the increased production of both pro- and anti-inflammatory cytokines. Together, these studies suggest that trophoblast cells can indeed function similarly to cells of the innate immune system, by recognizing and responding to components of microorganisms.

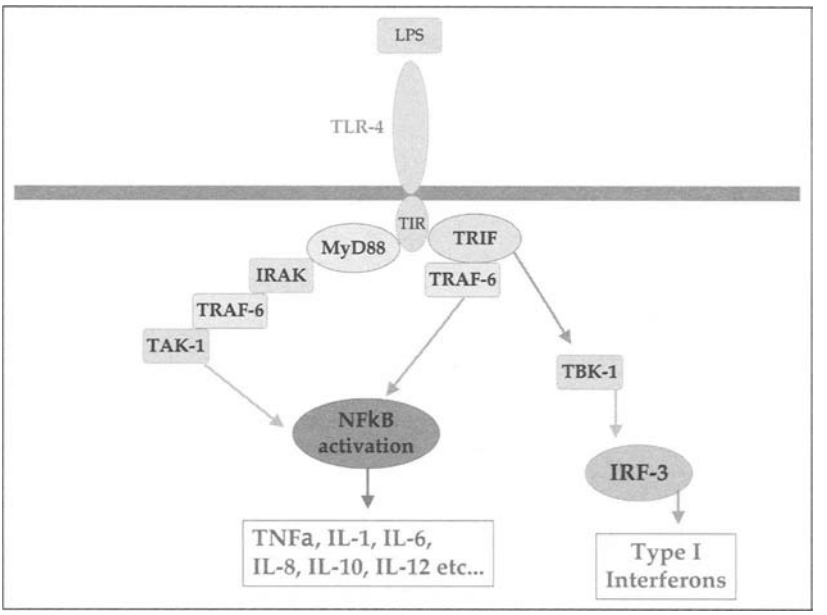

Figure 2. MyD88-dependent and -independent signaling of TLR-4. Activation of NF-κB by TLR-4 can occur through the classical MyD88 signaling pathway, or in a MyD88-independent manner though the recruitment of TRIF.

Toll-Like Receptors and Apoptosis

In many cases, infection can result in apoptosis and death of infected cells and this is an important aspect of host defense.[12] A striking finding from our studies has been the effect of peptidoglycan on first trimester trophoblast cell survival. Peptidoglycan is the major component of gram-positive bacteria and a ligand for TLR-2. We have found that ligation of TLR-2 by peptidoglycan fails to upregulate cytokine production by trophoblast cells. Instead, activation of TLR-2 induces first trimester trophoblast cells to undergo apoptosis. Aliprantis et al,[32] showed that both the monocyte cell line THP-1, as well as kidney epithelial cells transfected with TLR-2, undergo apoptosis following ligation of TLR-2 with bacterial lipoproteins. Similarly, Lopez et al,[74] showed that macrophages undergo TLR-2-mediated cell death in response to *Mycobacterium tuberculosis*. Interestingly from our studies, another TLR-2 ligand, lipoteichoic acid also induces trophoblast cell apoptosis. Recognition of peptidoglycan by TLR-2 requires the additional recruitment of TLR-6, while lipoteichoic requires TLR-1.[75,76] In first trimester trophoblast cells we have been able to detect mRNA for TLR-1 but not for TLR-6suggesting that in first trimester trophoblast cells, the apoptotic pathway may be activated through a heterodimer of TLR-2/TLR-1 or TLR-2/TLR-2 homodimer.

When we evaluated the mechanism of TLR-2 mediated apoptosis, our studies showed that the induction of TLR-2 mediated apoptosis occurs through activation of the caspases and that initiation of this intracellular pathway is dependent upon the recruitment of Fas-associated death domain (FADD) by MyD88 (Fig. 3). These results are in agreement with the recent findings in myeloid cells.[77,78] Furthermore, a recent report has shown that TLR-3 mediated apoptosis also occurs in a FADD-dependent, but MyD88-independent manner (Fig. 3). Overexpression of TRIF induces FADD-mediated apoptosis, however, TRIF lacks a death domain and cannot directly interact with FADD to activate the caspase cascade. Instead, TRIF can induce FADD-mediated activation of the apoptotic pathway via the

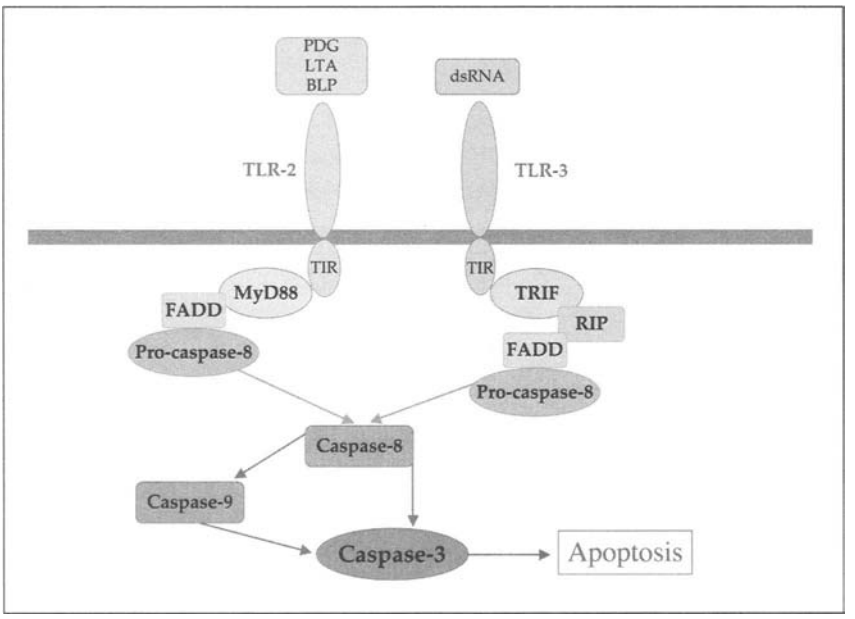

Figure 3. Induction of apoptosis by Toll-like receptors. Activation of the apoptotic caspase cascade by FADD in either a MyD88-dependent manner following ligation of TLR-2, or by a MyD88-independent pathway following ligation of TLR-3.

interaction of TRIF with RIP.[71] Whether TLR-3 can mediate trophoblast cell apoptosis is, as yet, unknown.

Infection, Toll-Like Receptors and Pregnancy Complications

Disturbances in the regulation of apoptosis within the placenta appears to be associated with abnormal pregnancy outcome.[79] Elevated trophoblast apoptosis is seen during the first trimester of pregnancies complicated with IUGR or preeclampsia[80-82] and this is accompanied by reduced trophoblast invasion and spiral artery transformation.[83] Furthermore, elevated trophoblast apoptosis has been observed in preterm births.[84,85]

Since clinical studies have shown an association between intrauterine infections and preterm labor, preeclampsia and IUGR,[4-8] we hypothesize that Toll-like receptors expressed at the maternal-fetal interface may play an important role in the mechanism of pathogenesis. We predict that certain intrauterine infections during pregnancy may have either a direct or indirect effect upon trophoblast cell survival, depending upon which TLR is activated. A gram-positive bacterium expressing peptidoglycan or lipoteichoic acid may directly promote trophoblast cell death though TLR-2 (Fig. 4). Recently soluble TLR-2 has been identified.[86] This protein may function by modulating specific TLR-mediated responses. Alternatively, soluble forms of TLR may bind to microorganisms and flag them for destruction by the complement system or by phagocytosis.[12] Soluble Toll-like receptors may, therefore, provide new markers of pregnancy complications as well as a potential target for therapeutic interventions.

Animal models of pregnancy complications have been generated by the administration of gram-negative bacterial LPS.[87-91] LPS, through TLR-4, triggers first trimester trophoblast cells to produce high levels of cytokines, including TNFα and IFNγ. We and others have shown

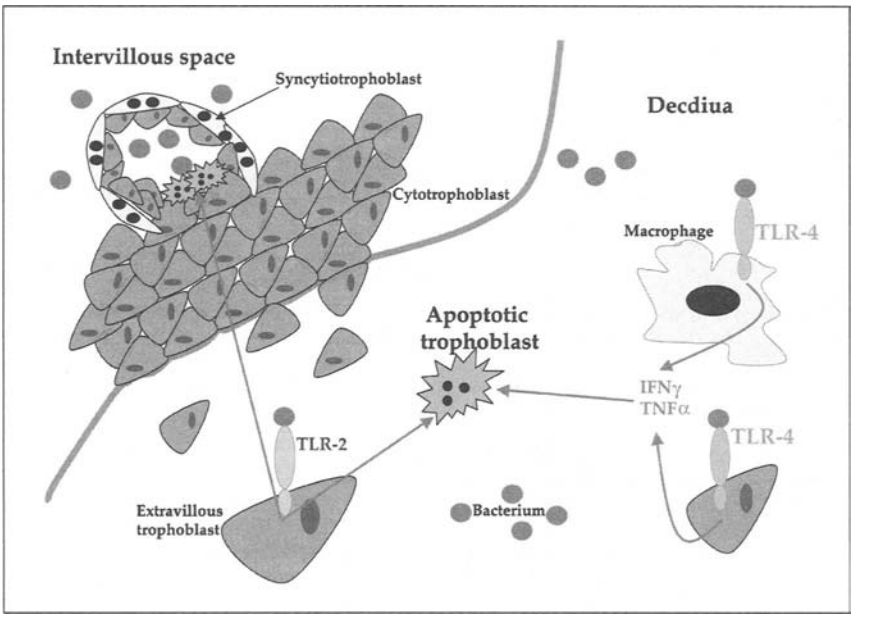

Figure 4. A model for the induction of trophoblast apoptosis in pregnancies complicated by infections. Apoptosis of cytotrophoblast or extravillous trophoblast cells may occur directly through activation of TLR-2. Alternatively, elevated trophoblast apoptosis may be triggered indirectly by pro-inflammatory cytokines produced by TLR-4 expressing immune cells or trophoblast cells.

that trophoblast cells are highly sensitive to these cytokines, suggesting that TNFα and IFNγ expression in the placenta may induce trophoblast cell apoptosis.[92-96] Therefore, while LPS does not directly induce trophoblast cell death, the intense inflammatory response generated by either trophoblast or decidual immune cells following its activation may provide an alternative mechanism for the induction of trophoblast cell death (Fig. 4).

Summary

There is growing evidence that trophoblast cells are able to recognize and respond to pathogens through the expression of Toll-like receptors, a system characteristic of innate immune cells. Interestingly, activation of different TLR appears to generate distinct trophoblast cell responses. We have found that TLR-4 ligation promotes cytokine production, while ligation of TLR-2 induces apoptosis in first trimester trophoblast cells. These findings suggest that a pathogen, through TLR-2, may directly promote the elevated trophoblast cell death observed in a number of pregnancy complications. TLR-2 mediated trophoblast apoptosis, therefore, provides a novel mechanism of pathogenesis by which certain intrauterine infections may contribute to conditions such as preterm labor, IUGR and preeclampsia. How the immune system functions during early pregnancy remains an uncertain area. The field of Toll-like receptors represents an exciting area of innate immunity and it is becoming increasingly clear that Toll-like receptor signaling can generate distinct immunological outcomes. The expression and function of Toll-like receptors at the maternal-fetal interface is a novel area of reproductive immunology with much need for future studies.

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CHAPTER 3

IL-10 and Pregnancy

Shaun P. Murphy and Surendra Sharma

Introduction

Soon after the principles of nonself immunological recognition were discovered, it was realized that the state of pregnancy seemingly presents a paradox. In an outbreed population, half of the fetal genes are paternal, thus the fetus may be considered a semi-allograft. Yet, unlike the outcome of a surgical tissue graft, the mother tolerates and nurtures the fetus.

Research into this problem has yielded deeper insights into the immunology of pregnancy. The pregnant uterus and the site of a surgical tissue graft may be thought of as analogous, but they exhibit important dissimilarities. Both sites express pro-inflammatory cytokines such as IFN-γ and IL-1β. These cytokines mediate anti-graft responses and have been shown to adversely affect pregnancy. However, unlike the site of a tissue graft, the maternal-fetal interface also expresses many anti-inflammatory cytokines and other factors that limit immunological aggression towards the fetus. It is thought that the balance of locally produced pro-inflammatory and anti-inflammatory cytokines is critical to the success of pregnancy.[13,27] Among these locally-produced factors, IL-10 seems to be the most potent immunosuppressive and anti-inflammatory molecule. First discovered as a molecule that could inhibit cytokine production and proliferation of T cells,[22] IL-10 has been shown to exhibit a wide array of immunosuppressive activities on various immune cells. Among these include the inhibition of antigen presenting cell function, inhibition of expression of inflammatory cytokines, inhibition of cytotoxic T cell (CTL) responses and induction and function of regulatory T cells, and regulation of the survival and proliferation of B cells.[56,48,88] Additionally, IL-10 has been shown to both inhibit and promote the growth of tumors.[53] IL-10 is known to downregulate MHC class I expression on tumor cells, thus inhibiting CTL killing of these cells. However, downregulation of MHC class I molecules might render tumor cells susceptible to NK cell killing. In addition, subpopulations of regulatory T cells have been shown to produce IL-10 to regulate inflammatory responses. Interestingly, a recent report suggests that $CD4^+CD25^+$ T cells expand during pregnancy and promote successful pregnancy outcome.[3] It remains to be seen whether pregnancy-associated regulatory T cells exert their function via IL-10.

IL-10 Gene, Protein, and Expression

The protein and gene structure of both human (hIL-10) and mouse (mIL-10) share a high degree of homology. The cDNA clones of hIL-10 and mIL-10 display greater than 80% nucleotide sequence homology, the inclusion of a human *Alu* repetitive sequence in the 3'-untranslated region of the hIL-10 cDNA being the only major disparity.[54,86]

The protein products of hIL-10 and mIL-10 are quite similar, exhibiting 73% amino acid homology. Human IL-10 is an 18kDa polypeptide lacking N-glycosylation sites. T cell-derived mIL-10 exists as three heterogeneously glycosylated proteins of 17, 19, and 21 kDa.[57] Glycosylation has no apparent effect on the biological activity of mIL-10 as mutant proteins

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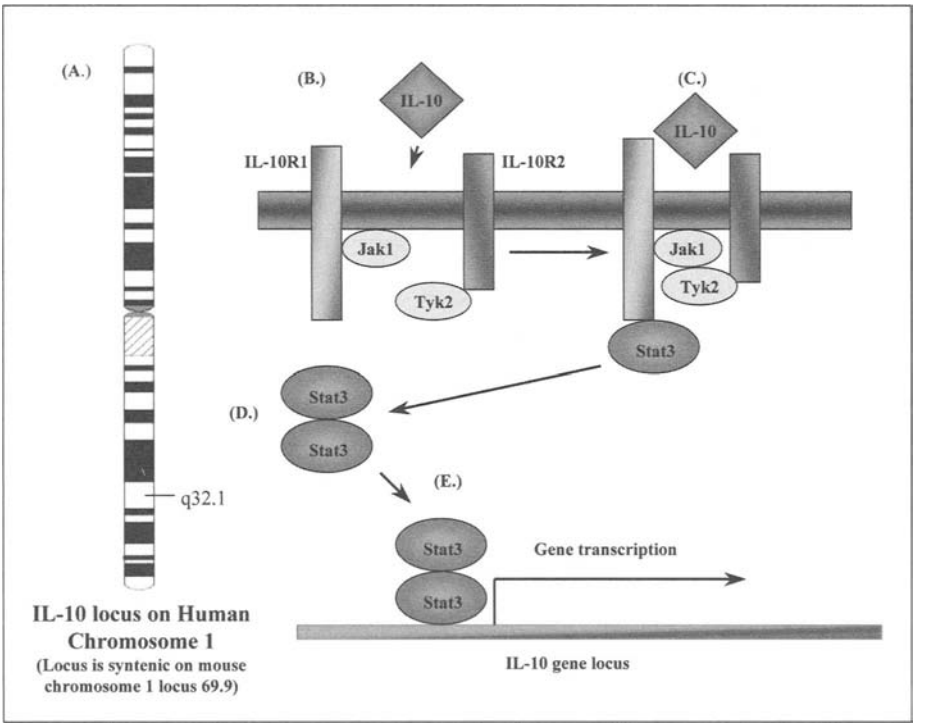

Figure 1. The location of the IL-10 gene on human and mouse chromosome 1 (A). Signal transduction of IL-10 begins with ligand binding to the IL-10 receptor (B). This leads to activation of the Jak1 and Tyk2 kinases and their phosphorylation of IL-10R1, which then recruits Stat3 (C). Jak1/Tyk2 phosphorylates the bound Stat3 leading to Stat3 homodimerization (D). The Stat3 dimers translocate to the nucleus, and drive the expression of IL-10-induced genes (E).

lacking N-linked glycosylation sites retain biological activity. mIL-10 has not been shown to be biologically active on human cells, although hIL-10 is active on cells of both species.

Both human and mouse IL-10 genes are on chromosome 1 of each species[34] (Fig. 1A). Transcriptional activation of the IL-10 gene results in ~2 kb (hIL-10) and ~1.4 kb (mIL-10) mRNAs. IL-10 is expressed in a wide variety of cell types. Among immune cell populations, IL-10 expression has been observed in T cells, B cells, macrophages, dendritic cells, and NK cells.[55,87] Many nonimmune cell populations also express IL-10; most prominently both mouse and human placental trophoblast cells and decidual stromal cells.[27,39,64,74,83]

Regulation of IL-10 gene expression has been associated with diverse pathological conditions. Although IL-10 transcriptional regulation is not currently well defined, Sp1 and Sp3 transcription factors are constitutively involved in many cell types. Importantly, the promoter region of the human IL-10 gene is known to harbor multiple polymorphic sites. Several reports have described the presence of three linked single nucleotide polymorphisms (SNPs) found at -1082(G/A), -819(C/T), and -592(C/A) base pairs upstream from the transcriptional start site.[19] These SNPs are in linkage disequilibrium and are thus inherited as haplotypes. The following major haplotypes of the -1082, -819, and -592 SNPs are found in the human population: GCC, ACC, and ATA.[93] These polymorphisms have been shown to have effect on IL-10 production in vitro.[84] Most studies suggest the GCC haplotype is associated with high in vitro IL-10 production and the ACC and ATA haplotypes are associated with low IL-10 production.[17,20,23,84] These haplotypes combine to form genotypes associated with high

expression (GCC/GCC), intermediate expression (GCC/ATA, GCC/ACC, and ACC/ACC) and low expression (ATA/ATA and ATA/ACC) of IL-10. However, regulation through these haplotypes can be altered by upstream polymorphic changes. For example, the distal A-A haplotype (-3575 and -2763) linked with the GCC haplotype has recently been shown to result in decreased IL-10 production and is associated with high incidence of graft versus host disease.[95]

Numerous studies have suggested an association between certain IL-10 promoter haplotypes and disease susceptibility or severity. A particularly well-studied example comes from patients suffering from systemic lupus erythematosis (SLE). Studies have shown a link between high IL-10 production and the etiology or exacerbation of the disease.[26,42,43] Evidence has also been found for the association of the high-production GCC haplotype with patients suffering from severe SLE accompanied by anti-SSA (Sjogren's syndrome A antigen) antibodies.[38]

Recently, associations between various other diseases and IL-10 promoter haplotypes and genotypes have been investigated. Adverse clinical outcome in patients suffering from primary progressive multiple sclerosis appears to be associated with the low IL-10 production ATA haplotype.[96] The ATA haplotype is also associated with spontaneous hepatitis C viral clearance[47] and susceptibility to aggressive nonHodgkin's lymphoma.[11] Additionally, there seems to be a link between the GCC/ACC genotype and Alzheimer's disease.[77] These important observations provide a basis for comprehensive study to link the IL-10 gene promoter polymorphisms with adverse pregnancy outcomes.

IL-10 Receptor and Signaling

Both human and mouse IL-10 signal through at least two receptor subunits, IL10R1 and IL-10R2. IL-10R1 seems to be the major subunit as monoclonal antibodies to IL-10R1 block all known biological activities of IL-10.[32,41,61] hIL-10R1 and mIL-10R1 are proteins of 578 and 576 amino acids respectively and share 60% homology. In accord with the species specificity of IL-10, mIL-10R1 binds both mIL-10 and hIL-10 while hIL-10R1 does not bind mIL-10.[32]

The expression of IL-10R1 has been observed in most hematopoetic cells[80] and in several nonhematopoeitc cell types. Cytotrophoblasts[27,75] and intestinal epithelial cells[6,15] constitutively express IL-10R1, while expression in fibroblasts, epidermal cells, and keratinocytes is inducible.[50-52,89]

IL-10R2 appears to be an ancillary subunit to IL-10R1. Although cells from IL-10R2$^{-/-}$ mice do not respond to IL-10,[56] mIL-10R2 does not bind mIL-10 but appears to be required for recruitment of downstream signal transduction molecules.[36,78] The expression profile of IL-10R2 mirrors that of IL-10R1, although it is constitutive.[24,44]

The major IL-10R signal transduction pathway appears to crucially involve the Jak/stat system (Fig. 1B-E). The tyrosine kinase Jak1 is constitutively bound to IL-10R1[85] and this association seems necessary for signaling as Jak1$^{-/-}$ mice are unresponsive to IL-10.[72] Another member of the Jak kinase family, Tyk2, is constitutively bound to IL-10R2.[35] Upon ligation of IL-10 by the IL-10R, Jak1 and Tyk2 are activated[21] and phosphorylate IL-10R1.[90] Phosphorylated IL-10R1 recruits Stat3, triggering the phosphorylation of Stat1, Stat3, and Stat5 by Jak1/Tyk2, the hetero- and homodimerization of Stat1, Stat3, and Stat5,[91] and ultimately translocation of the Stat dimers to the nucleus to drive the transcription of IL-10-induced genes.

The significance of Stat1 and Stat5 in IL-10 signal transduction is unclear. Neither Stat1 nor Stat5 interact with the IL-10R complex,[90] inhibition of Stat1 or Stat5 function has no apparent effect on IL-10 responses,[61] and macrophages isolated from mice genetically deficient in Stat1 are able to respond to IL-10.[46] However, Stat3 appears to be necessary for known IL-10 functions in all studied cells.[56]

IL-10 Expression during Pregnancy

Abundant expression of IL-10 can be found in various cells at the maternal-fetal interface. Both human and mouse placental tissue expresses IL-10 in a gestational age-dependent manner, with strong expression in early and mid-pregnancy, declining at term.[5,9,27,74,83] Expression

of IL-10 is also observed in normal human and mouse decidua, at least during early pregnancy stages.[14,33,39,64,83] In addition to placental and decidual expression, IL-10 is also expressed by a variety of hematopoetic cells found at the maternal-fetal interface, including macrophages, NK cells, and T cells.[18,28,40,60,87] The expression of IL-10R largely mirrors that of its ligand at the maternal fetal interface with expression in both the placenta and decidua, although expression appears to persist in both tissues until term.[27]

Functions of IL-10 at the Maternal-Fetal Interface

Evidence that IL-10 plays a role in the maintenance of pregnancy was first gleaned from a mouse model of spontaneous abortion. Mating between the inbred mouse strains CBA and DBA/2 results in higher rate of spontaneous abortion than mating between CBA and another inbred mouse strain, BALB/c. This increased spontaneous abortion rate is accompanied by a decrease in the production of IL-10 from both the decidua and the placenta. Additionally, administration of recombinant IL-10 (rIL-10) to the female mice in this mating model reduces the rate of abortion.[10]

Cytotrophoblast invasion of the uterus is crucial for the establishment of an efficient fetal-maternal vascular exchange. While the invasion of the cytotrophoblast must be of a sufficient depth to ensure the establishment of proper nutrient and waste exchange, it must not be so deep as to cause harm to the mother. Along these lines, several studies also suggest that IL-10 expression may regulate the process of placentation. Major mediators of trophoblast invasion are members of the matrix metalloproteinase (MMP) family, which are collagenases that degrade the extracellular matrix. Cytotrophoblast invasion in an in vitro model is associated with increased expression of MMP-9. Isolated cytotrophoblasts in this model rapidly acquire an invasive phenotype, which is associated with downregulation of IL-10 expression and upregulation of MMP-9 expression. IL-10 is a known mediator of MMP activity in various models of tumor metastasis through its ability to induce expression of various members of the tissue inhibitor of metalloproteinase (TIMP) class of molecules.[79] Furthermore, the addition of rIL-10 to cytotrophoblast cultures results in the downregulation of MMP-9 expression and the reduction of cytotrophoblast invasiveness.[75] TIMPs are expressed at the maternal-fetal interface and these molecules are likely to regulate MMP-mediated trophoblast invasion.[2,76]

In response to blastocyst implantation in the uterine cavity, the cytotrophoblast comes into direct contact with specialized bone marrow-derived immune cells. It is believed that chemotactic activity of cytotrophoblasts as a result of chemokine expression preferentially recruits NK cells, monocytes, and T cells.[16] Although the focus on immune cell expansion during pregnancy has been on the local immune cell population, it is also possible that the systemic expansion of these specialized cells might also occur, as is the case in mice with regulatory T cells ($CD4^+CD25^+$).[3] The major location of immune cells at the maternal-fetal interface is the decidua. Although the decidua is populated early during pregnancy with a variety of immune cell populations in various proportions, the major population of immune cells at the site of placental attachment to the mother is of the NK cell lineage. These uterine NK (uNK) cells migrate into the human uterus during the proliferative phase of the menstrual cycle in humans, or in response to implantation in mice. A majority of human uNK cells highly express CD56 but lack expression of CD16, a hallmark of peripheral NK cells. They increase in number as pregnancy progresses, peak during mid-gestation and gradually diminish in number and are nearly absent at term.

A hallmark of NK cell function is the so-called "missing-self" killing hypothesis, whereby NK cells are inhibited from killing cells that express MHC class I molecules, but actively kill cells lacking such expression. As trophoblasts lack expression of classical MHC class I molecules, they would be expected to be prime targets for NK cell cytotoxicity. However, human trophoblasts express the nonclassical, relatively nonpolymorphic MHC class I molecule HLA-G, which inhibits NK cell cytotoxicity. Expression of class I and class II MHC molecules is downregulated by IL-10 in most cells studied. However, treatment of isolated human

trophoblast and monocytes with IL-10 induces HLA-G expression, suggesting a role for IL-10 in fetal protection from NK-cell mediated cytotoxicity.[58]

The functional significance of uNK cells remains largely obscure. Recent observations in mice suggest that uNK cells might regulate the transformation of decidua and spiral artery remodeling via IFN-γ production. On the other hand, mice lacking NK cells or IFN-γ signaling experience normal pregnancy, particularly in multiparous females. Curiously, these cells present a paradox in that they express high amounts of perforin yet are not cytotoxic during normal pregnancy. The mechanisms that might activate these cells to become cytotoxic during pregnancy are not understood.

Recently, mice genetically deficient in IL-10 have been available, allowing the direct study of IL-10 deficiency on pregnancy outcome. While these mice are fertile under pathogen-free conditions, they exhibit certain placental abnormalities. Compared to wild type mice, the placenta of IL-$10^{-/-}$ mice are comparatively larger in size and blood space, and this was associated with an increased cytotrophoblast invasiveness. Pups from these mating have an increased birth weight, consistent with more efficient placental function.[71] However, pups delivered by IL-$10^{-/-}$ mice exhibit postnatal growth impairment well into adulthood.[97]

While IL-10-deficient mice are fertile when mated in pathogen-free conditions, their pregnancy outcome appears to be acutely sensitive to inflammatory insults. Lipopolysaccharide (LPS), an endotoxin derived from gram negative bacteria, is a potent inducer of abortion and preterm birth in murine models. Our unpublished results point to the fact that treatment of pregnant IL-$10^{-/-}$ mice with LPS at doses too low to influence pregnancy in wild type mice causes fetal demise in mice. This increase in the sensitivity of IL-$10^{-/-}$ mice to the abortive effects of LPS appears to be associated with an increase in uterine natural killer cell activity (Murphy and Sharma, manuscript in preparation).

IL-10 has also been implicated in the regulation of prostaglandin synthesis. Prostaglandin E_2 (PGE_2) and prostaglandin $F_2\alpha$ (PGF_2) are produced by fetal membranes and are thought to play a role in parturition. The major regulatory step in the prostaglandin synthesis pathway is considered to be mediated by the inducible enzyme cyclooxygenase-2 (COX-2). Treatment of human fetal membrane tissues and placental explants with IL-10 reduces their COX-2 expression and PGE_2 production[8,65] (Hanna and Sharma., manuscript in preparation). This finding, coupled with the observation that placental IL-10 production is downregulated at term[27] suggests a role for IL-10 in the preservation of pregnancy via inhibition of labor until gestation has completed.

Pregnancy Pathologies Associated with Abnormal IL-10 Expression

Recurrent Spontaneous Abortion

Recurrent spontaneous abortion (RSA) is defined as the occurrence of three or more spontaneous abortions and occurs in about 2-4% of women.[1] About 50% of RSA cases can be attributed to chromosomal anomalies, endocrine disorders, or anatomical abnormalities.[7,37,73] Immunological explanations have been sought to explain the remaining 50% unexplained RSA. Recent evidence in humans suggests that perturbations in the balance of pro- and anti-inflammatory cytokine expression is associated with RSA. Women with a history of RSA exhibit decreased IL-10 expression in peripheral blood mononuclear cells (PBMC).[45,66,67] However, these observations are accumulated in response to PBMC activation by polyclonal or recall antigens and do not address the status of local IL-10 production. Our recent work clearly demonstrated a lack of IL-10 production in decidual and placental tissue from patients with spontaneous abortion.[64] In addition, serum cytokine levels of IL-10 appear lower in women suffering from RSA than women experiencing normal pregnancy.[46]

It may be expected that IL-10 promoter polymorphisms may influence pregnancy outcomes, with low-production phenotypes perhaps associated with increased risk of pregnancy abnormalities. Several studies have investigated this potential role in the etiology of RSA,

yielding conflicting results.[4,12] However, it should be understood that cytokines often influence and modulate the expression and functions of other cytokines. Pro- and anti-inflammatory cytokines counter-regulate each other such that the balance between their expression is crucial, not their individual expression levels. Women with IL-10 high-production promoter haplotypes that experience RSA may also have high-production promoter haplotypes of pro-inflammatory cytokine promoters or are otherwise prone to severe inflammation. The converse may be true for women with low IL-10-production promoter haplotypes that experience normal pregnancies.

Preeclampsia

The hallmark symptoms of preeclampsia are transient hypertension, proteinurea, and edema. It is thought that a major cause of preeclampsia is shallow trophoblast invasion, resulting in poor maternal-fetal vascular exchange and ultimately ischemia. While this disease is believed to have a myriad of causes, reduced IL-10 production has been observed in the placenta, decidual lymphocytes, and PBMCs from women suffering from preeclampsia.[29,62,98] Considering the role that IL-10 plays in the inhibition of MMPs required for trophoblast invasion, it is odd that reduced IL-10 would be observed in a disease of inadequate trophoblast invasion. However, various factors, including the inflammatory cytokines IFN-γ and TNF-α have been shown to inhibit trophoblast migration and also to be directly cytotoxic.[68,82,94] IL-10 can counteract the effect of these cytokines and may protect the trophoblast from their cytotoxic effect. Indeed our preliminary data suggests that there is a soluble factor in the serum from many preeclamptic women that induces apoptosis to trophoblast in vitro. Treatment of trophoblasts exposed to preeclamptic serum with rIL-10 appears to prevent their apoptosis (Sharma, manuscript in preparation). Thus, IL-10 may serve a dual function as a trophoblast survival factor as well as an anti-inflammatory cytokine. Interestingly, there is some evidence that preeclampsia may be associated with increased placental IL-10 expression.[69] This result is perhaps not so surprising owing to the heterogeneous nature of preeclampsia.

Preterm Birth and Intrauterine Growth Restriction (IUGR)

Normal term labor is associated with an increase in the production of various inflammatory mediators by the fetal membranes and myometrium including IL-1β, IL-6, IL-8, TNF-α, PGE_2. In addition, there is an increase in the expression of COX-2 and MMP-9, and a decrease in TIMP.[25,99] Conversely, local IL-10 expression is attenuated at term,[27] consistent with the role of IL-10 as a negative regulator of these inflammatory stimuli.

A major cause of preterm labor is thought to be infection, especially local infection at the maternal-fetal interface. Infection is detected in over 30% of preterm labor cases, with ureaplasma, urealyticum, mycoplasma hominis, streptococcus agalactae, and escherichia coli the most common organisms isolated.[30,31] This intrauterine infection may result in a premature induction of an inflammatory cytokine cascade similar to that observed in normal labor.

This intrauterine infection hypothesis of preterm birth has been investigated in various animal models. Rats injected with LPS late in gestation experience preterm birth and delivered pups with below normal birth weights. However, intra-uterine administration of IL-10 at the time of LPS treatment resulted in pups delivered at full term with normal birth weights.[81] Additionally, as noticed above, mice deficient in IL-10 appear to be more sensitive to LPS-induced preterm labor. LPS administered to IL-10 deficient mice late in gestation results in premature labor whereas the same dose of LPS has no effect on pregnancy in wild type mice (Murphy and Sharma, manuscript in preparation).

IUGR is also thought to be associated with inflammation or infection of the uterine microenvironment. It has been demonstrated that IL-10 might attenuate fetal growth restriction and associated demise.[70] Indeed, differential effects of LPS administration at different doses in pregnant IL-$10^{-/-}$ mice include IUGR and preterm birth (Murphy and Sharma, manuscript in preparation).

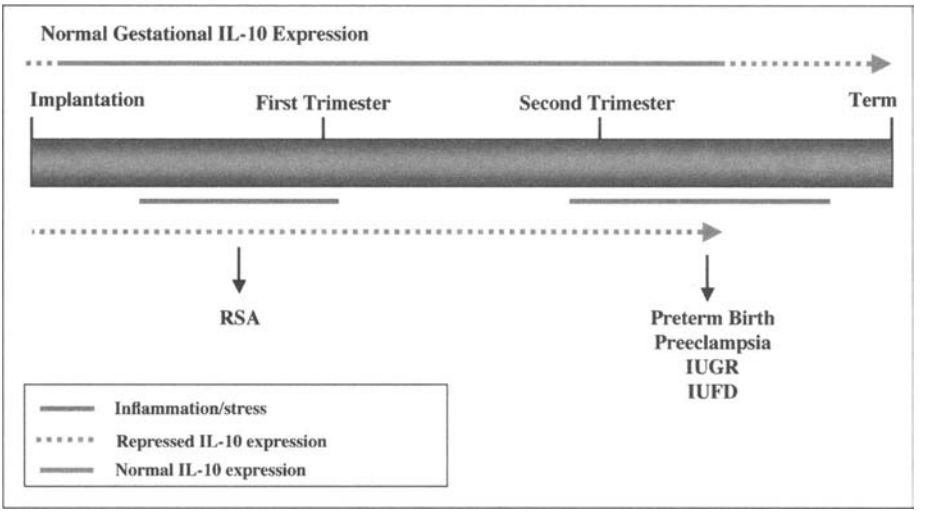

Figure 2. Effect of inflammation or aberrant temporal expression of IL-10 on the occurrence of pregnancy disorders. IL-10 expression at the maternal fetal interface may begin immediately after implantation and persist well into the early part of third trimester. Predisposition to low IL-10 production, inflammation, or both can trigger an unbalanced pro-inflammatory/anti-inflammatory milieu leading to various maladies of pregnancy. If this occurs early in pregnancy in multiple pregnancies, RSA may result. A later occurrence of these events may lead to such conditions as preeclampsia, intrauterine growth restriction (IUGR), intrauterine fetal demise (IUFD), or preterm birth.

Conclusions

Taken together, the body of research discussed herein suggests a paradigm for the role of IL-10 during pregnancy. While IL-10-deficient mice are fertile, it must be emphasized that these animals are typically bred under pathogen-free conditions as they mount exaggerated inflammatory response to pathogens. Whereas IL-10-proficient mice are perfectly fertile in "normal" conditions, where no care is taken to protect them from environmental pathogens, IL-10-deficient mice experience decreased fertility when bred under the same conditions. As pro-inflammatory factors are well-known inducers of fetal demise in mice, the absence of expression of the anti-inflammatory cytokine IL-10 may result in a local, at the maternal-fetal interface, and/or systemic immune deregulation, leading to adverse pregnancy outcome depending on the time of the onset of inflammation. Although IL-10 nonproducers have not been identified in the human population, reduced IL-10 production has been associated with increased risk of several disorders of pregnancy. It is tempting to speculate that many cases of pregnancy anomalies, especially preterm birth, preeclampsia, and RSA may be secondary to local, often subclinical infections. Women with reduced IL-10 production may be especially sensitive to the inflammatory effects of subclinical infection, resulting in adverse pregnancy outcomes (Fig. 2). However, women with adequate IL-10 production may be able to limit the consequences of inflammatory response to a subclinical level, allowing the maintenance of pregnancy.

Acknowledgements

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CHAPTER 4

Th1/Th2 Balance of the Implantation Site in Humans

Shigeru Saito, Satomi Miyazaki and Yasushi Sasaki

Introduction

Successful embryo implantation requires the synchronization of embryo development and uterine preparation. The embryo must have developed to the blastocyst stage and the endometrium must be in a receptive phase. Wilcox et al[1] have estimated that 65% of conceptions end in unrecognized losses. These failures can be divided into failure to implant (20%), initial apposition but no adhesion or invasion (28%), and failure to develop immediately after implantation (17%). After implantation, approximately 15% of human conceptions end in a clinically detected spontaneous abortion. Furthermore, approximately 31% of pregnancies are lost after detection using sensitive assays for the beta subunit of human chorion gonadotropin (β-hCG). Therefore, implantation failure and pregnancy loss are the most common complications of pregnancy. Understanding the molecular factors involved in each phase of implantation and early pregnancy is critical to understanding the mechanism controlling reproduction.

Human pregnancy represents a semiallograft to the maternal host. It is very interesting that the semi-allogeneic embryo/fetus is not rejected by the mother. Recent studies suggest that endometrial (maternal) lymphocytes play some roles in the maintenance of pregnancy via immune mediators such as cytokines.[2,3] Thus, the process of implantation should include mechanisms preventing allograft rejection. However, the absence of these regulatory factors may be involved in multiple implantation failure or pregnancy loss.

The relative by low rate of successful implantation in humans suggests that the expression of these cytokines and their biologic signals must be optimal, precise and synchronized. Little is known about the molecular markers that are essential for implantation and maintenance of pregnancy. However, in recent years, accumulating evidence has emerged that implicates many factors including cytokines, growth factors and maternal lymphocytes for embryo implantation. In this chapter, I focus on the role of immune cells, especially Th1 and Th2 cells, in the reproductive phenomenon during early pregnancy.

T Cells Change the Implantation Window and Promote Embryo Implantation in Mice

The blastocyst and the maternal endometrium develop an exquisite dialogue during the so-called 'implantation window', which allows them to complete the implantation process. In mice, the period of time begins at day 3 and is complete by day 5. In humans, days 20 to 24 of the regular 28-day menstrual cycle are the optimal period for implantation. Pseudopregnancy at day 2 in mice does not represent the receptive phase for implantation of embryos. Takabatake et al[4] reported that recipient pseudopregnant ICR or BALB/C mice were injected intravenously with splenocytes or culture supernatant on day 2, and blastocyst transfer was formed on

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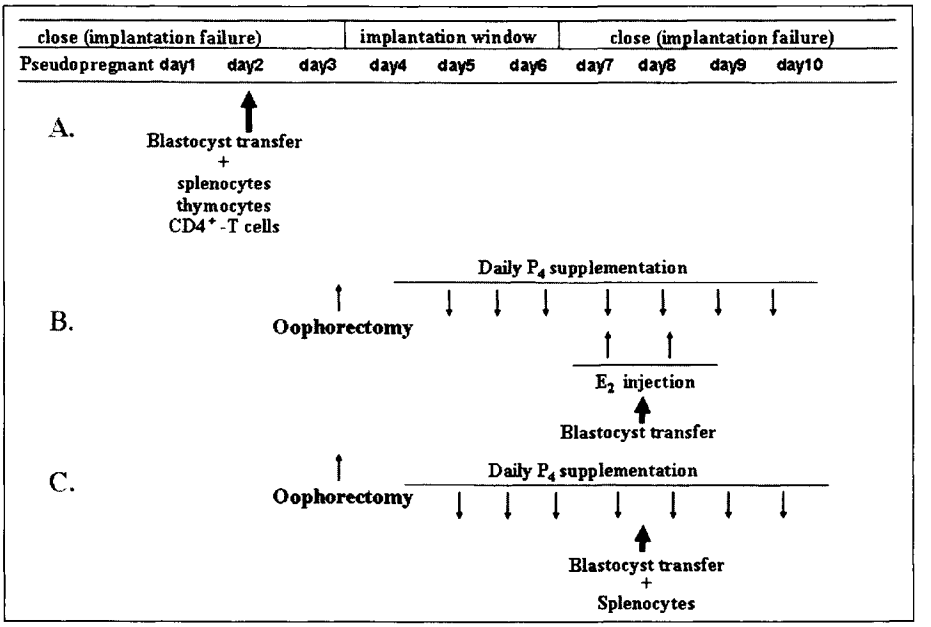

Figure 1. Schedules of embryo transfer experiments.

day 2 (Fig. 1A). The successful implantation rate was markedly high in the pregnancy day 4- and day 8 splenocyte-injected groups. However, the implantation rate was low when di-estrous splenocytes were injected into recipient pseudopregnant mice. Interestingly, the supernatant of pregnancy day 4 and day 8 splenocytes could not improve the implantation rate. These data suggest that peripheral immunocompetent cells in early pregnancy may be involved in embryo implantation, and humoral factors in culture supernatant are not sufficient for changing the implantation window. They further clarified that a significant increase in the implantation rate was observed when $CD4^+$ T-cell-rich pregnancy splenocytes were injected into the endometrial stroma of the unilateral uterus, while no significant increase was observed when monocyte-rich pregnancy splenocytes were injected into the uterus.[5] This suggests that T-lymphocytes during early pregnancy could contribute to changing the implantation window and the increase of implantation. T cells accumulated at the uterus or implantation site might regulate endometrial differentiation and embryo implantation under the control of cytokines at the materno-fetal interface. Their group also reported on interesting study.[5] As a model for delayed implantation, pseudopregnant ICR mice were ovariectomized on day 3, and received daily injections of progesterone from day 4 to day 7. Administration of 17 β-estradiol on pseudopregnancy days 7 and 8 is known to induce delayed implantation (Fig. 1B). They intravenously injected pseudopregnancy day 4 splenocytes into these mice (Fig. 1C). Under progesterone supplementation, successful implantations were observed in the pseudopregnancy day 4 splenocyte-administer group without 17 β-estradiol-treatment group, but not in the splenocyte culture supernatant-treated group.

Among the cytokines so far examined, only the interruption of the leukemia inhibitory factor (LIF) gene results in failure of blastocyst implantation. Stewart et al[6] discovered that although the LIF knockout mice embryos displayed no defects and could implant in normal mice, the blastocyst remained free in the uteri of LIF-negative mice and no decidual reaction occurred. Thus, maternal expression of LIF is essential for successful implantation. Takabatake et al[5] reported that LIF mRNA was induced by pseudopregnant splenocyte

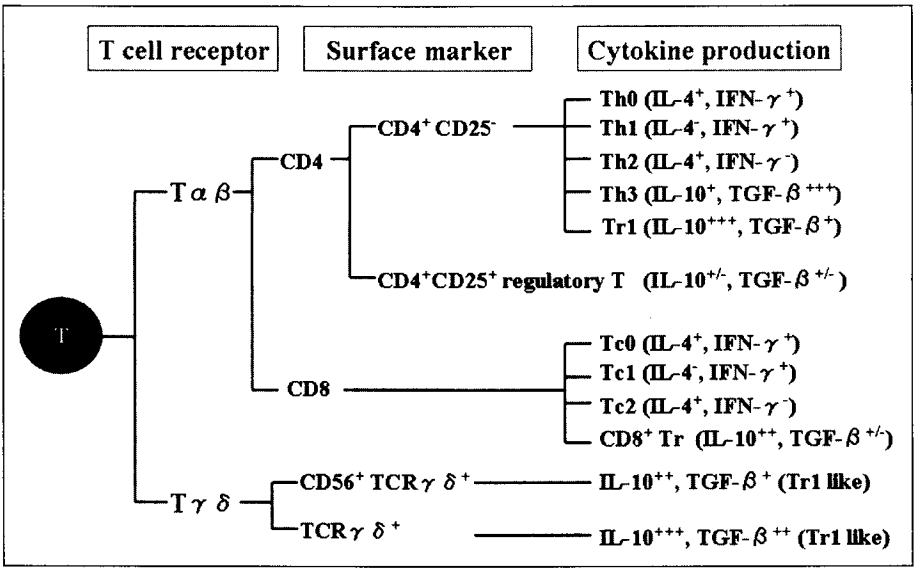

Figure 2. Classification of T cells by T-cell receptor, surface markers and cytokine production.

injection or 17-estradiol treatment in the delayed implantation system. These findings suggest that the effects of splenocytes on implantation are independent of the ovary, and the mechanism of the promoting effect of pseudopregnancy day 4 splenocytes on embryo implantation resembles that of estradiol. These studies demonstrated that implantation processes such as endometrial differentiation and embryo implantation might be carried out under the dual control of the endocrine and immune systems. Their group also reported that administration of thymocytes (T cells) derived from non-pregnant mice induced an endometrial receptive stage and LIF expression in the uterus.[7] Also, administration of $CD4^+$ T cells significantly promoted implantation rates, but no effect was observed in the $CD8^+$ T cell-injected group.[7] These findings showed that thymocytes, especially $CD4^+$ T cells, facilitate embryo implantation, probably by regulating endometrial differentiation.

Immunocompetent Cells in Human Endometrium and Early Pregnant Decidua

Lymphocyte Subsets

Lymphocytes are classified into T cells, NK cells, NKT cells, B cells and dendritic cells. T cells can be classified by T-cell receptors, surface markers and cytokine production (Fig. 2). T cells are classified into Tαβ cells and Tγδ cells by T-cell receptors. Tαβ cells are the main population in peripheral blood and endometrium, but Tγδ cells have some roles in the maintenance of pregnancy. Tαβ cells can be further classified into $CD4^+$ T cells, called helper T cells, and $CD8^+$ T cells, called cytotoxic T cells. Recently, $CD4^+CD25^{bright}$ regulatory T cells have been developed.[8] These regulatory T cells have a unique nature, regulating immunostimulation and inducing peripheral tolerance. About 10% of $CD4^+$ T cells are $CD4^+CD25^+$ regulatory T cells and 90% of other $CD4^+$ T cells are common helper T cells. Mosmann et al[9] proposed that $CD4^+$ helper T cells can be classified into Th0-, Th1- and Th2-type cells according to the types of cytokines they produce. After activation, naive T cells diffentiate to Th0 cells, which produce both Th1- type cytokines and Th2- type cytokines. Th0 cells further differentiate to Th1 cells or Th2 cells. Development of Th1- and Th2- type

responses depends on the cytokines produced in the microenvironment during antigen presentation. Interferon (IFN)-γ, IFN-α and interleukin (IL-12) promote the differentiation of naïve T cells, or Th0 cells, into Th1 cells, whereas IL-4 is the most dominant factor for Th2 polarization. $CD8^+$ T cells are also classified into Tc0, Tc1 and Tc2 cells by their cytokine production. Type 1 cells (Th1 cells and Tc1 cells) produce IFN-γ, IL-2 and tumor necrosis factor (TNF)-β, and induce cytotoxic T cells. Type 2 cells (Th2 cells and Tc2 cells) produce IL-4, IL-5, IL-6 and IL-13, and induce immunoglobulin production.

Recently, a new type of T cells called Th3 cells and Tr1 cells has been found, which produces both immunosuppressive cytokines, IL-10 and transforming growth factor (TGF)-β (Fig. 2). Th3 cells predominantly produce TGF-β, whereas Tr1 cells predominantly produce IL-10. The regulatory T cells such as Th3 cells, Tr1 cells and regulatory T cells play very important roles in immunosuppression and tolerance. It has been reported that decidual Tγδ cells resemble Tr1 cells in their cytokine production pattern.[10] NK cells are classified into $CD16^+CD56^{dim}$ NK cells and $CD16^-CD56^{bright}$ NK cells by their surface markers. Interestingly, the main population of peripheral blood NK cells is $CD16^+CD56^{dim}$ NK cells, whereas the main population of endometrial and decidual NK cells is $CD16^-CD56^{bright}$ NK cells.[11] Recent studies have demonstrated that $CD16^+$ NK cells and $CD16^-CD56^{bright}$ NK cells can be classified into NK1 cells, which produce Th1-type cytokines, and NK2 cells, which produce Th2-type cytokines, by their cytokine pattern.[12] NKT cells possess the T-cell marker CD3 and the NK-cell marker CD161. They recognize lipid antigen and have some roles in innate immunity. B cells are professional cells that produce immunoglobulin. In this process, Th2-type cytokines induce B-cell activation and differentiation. Dendritic cells (DC) are professional cells in antigen presentation. The activation and maturation of DC are key processes in directing naïve T cells to differentiate into Th1, Th2 and Tr cells.

Lymphocyte Subsets and Monocytes in Peripheral Blood, Endometrium and Early Pregnancy Decidua

The population of endometrial lymphocyte subsets rapidly varies throughout the menstrual cycle, although that in peripheral blood does not change dramatically (Table 1). There are at least two types of NK cells, $CD16^+$ NK cells and $CD16^-CD56^{bright}$ NK cells. Although $CD16^+$ NK cells are regarded as the main component of NK cells in peripheral blood, their content in the decidua is only 2%. In lymphocyte subsets, the number of $CD16^-CD56^{bright}$ NK cells gradually increases during the mid-luteal phase and, at its peak, accounts for up to 70% of lymphocytes in the early pregnancy decidua. These cells express a large amount of CD56, an NK marker, but they do not express any other NK- markers, such as CD16 or CD57. Interestingly, they number very few (~1%) in peripheral blood. There is a dense infiltrate of $CD16^-CD56^{bright}$ NK cells in the decidua basalis, where the implanting trophoblast cells infiltrate maternal tissues, suggesting that $CD16^-CD56^{bright}$ NK cells make a large contribution to implantation and have some roles in the maintenance of pregnancy. T cells are the main population of peripheral blood lymphocytes. Forty to 50% of endometrial lymphocytes in the proliferation phase are T cells, but they decrease to 30-35% in the secretory phase, and to 10-15% in the early pregnancy decidua. This decrease in the population of T cells is not caused by decreasing the number of T cells. The increasing number of $CD16^-CD56^{bright}$ NK cells causes the decreased ratio of T cells in total lymphocyte number. Many researchers have reported that it is unlikely that T cells can solely contribute to the maintenance of normal human pregnancy since they decrease significantly in the early pregnancy decidua. However, Michimata et al[13] reported that both the number and the ratio of T cells to lymphocytes are significantly higher in the decidua basalis than those in the decidua parietalis (see Table 1). Furthermore, both Th2 cells and Tc2 cells increase at the materno-fetal interface (implantation site) in the decidua. Also, a decrease in Th2 and Tc2 cells at the implantation site (decidua basalis) occurs in unexplained recurrent spontaneous abortion with normal chromosomal content, suggesting that T cells also contribute to implantation and maintenance of pregnancy.[14]

Table 1. Populations of lymphocytes and monocytes in uterine endometrium and peripheral blood

	Non-Pregnancy Endometrium		Early Pregnancy Decidua			Peripheral Blood
	Proliferation Phase	Secretory Phase	Decidua	Decidua Basalis	Decidua Parietalis	
T cell	40-50%	30-35%	10-15%	12%	6%	70%
$CD4^+$ T cell	15-20%	10-15%	5-7%	6%	3%	40-45%
$CD8^+$ T cell	20-25%	15-20%	7-10%	5%	3%	30-40%
Type 2 cell/T cell	0.5%	1.4%	15-20%	22%	16%	3%
Type 1 cell/T cell	60%	40%	10-15%	?	?	20%
NK cell						
$CD16^+$ T cell	2%	4%	2%	2%	2%	10-15%
$CD16^-CD56^{bright}$ NK	20%	40-50%	60-70%	69%	64%	<1%
NKT cell	?	?	0.4%	?	?	0.05%
B cell	~5%	~5%	2%	2%	2%	8-10%
Dendritic cell	?	?	1%	?	?	0.8%
Monocyte/macrophage	10%	10%	20%	30%	20%	8-10%

Takabatake's reports[4,5] that T cells promote embryo implantation in mice also suggest accumulated T cells at the decidua basalis may contribute to the maintenance of pregnancy by controlling Th1/Th2 balance.

The second major decidual leukocyte population consists of the monocytes/macrophages, which account for 20-30% of CD45-positive cells in first trimester decidua. Macrophages also increase at the decidua basalis (Table 1). Decidual macrophages have been implicated as suppressor cells by secretion of prostaglandin E_2. Another immunosuppressive enzyme, indoleamine 2,3-dioxygenase (IDO), is also expressed in macrophages.[15] IDO effects the suppression of T-cell activity by catabolizing the essential amino acid l-tryptophan. These findings suggest IDO in decidual macrophages might play an important role in the prevention of allogeneic fetal rejection at the materno-fetal interface.

As other lymphocyte subsets, NKT cells and dendritic cells are increased in early pregnancy decidua, compared to those in peripheral blood. However, the populations of these cells in the decidua are very low. The B cell count and the population in the endometrium do not change throughout menstruation and during pregnancy.

Th1/Th2 Balance in Normal Human Pregnancy

Th1-dependent effector mechanisms such as cytotoxic T lymphocyte (CTL) activity play a central role in acute allograft rejection. The production of Th2-type cytokines or regulatory cytokines such as TGF-β and IL-10 may be central to the induction and maintenance of allograft tolerance. Based on these findings, Wegmann et al[16] hypothesized that physiological protection from maternal rejection is due to a Th2-type response at the materno-fetal interface. In mouse models, Th1-type cells and CTL induce miscarriage. In humans, peripheral blood Th1/Th2 balance in normal pregnancy is controversial (Table 2), because methods of detection for Th1/Th2 ratio are different between papers and the amplitude of this balance is very small in peripheral blood. In our study, the Th1/Th2 ratio did not change in the first trimester of pregnancy.[17] The peripheral blood Th1/Th2 cell ratio in non-pregnant subjects is around 10-11, and these ratios decrease to 7-8 in the second and third trimesters.[18] Thus, the amplitude of the peripheral blood Th1/Th2 cell ratio during pregnancy is very little in human.

Table 2. Th1/Th2 balance in normal pregnancy subjects

Author	Samples	Methods	Results
Sabahi et al (1995)	PBMC	Cytokine secretion (PHA stimulation)	Th1 ↓
Marzi et al (1996)	PBMC	Cytokine secretion (PHA stimulation)	Th1 ↓, Th2 ↑
Jarvis et al (1996)	PBMC	RT-PCR (co-cultured with choriocarcinoma JAR)	Type 1 cytokines: IL-2 ↓, IFN-γ ↑ Type 2 cytokines: IL-4 →, IL-10 ↓
Ekerfelt et al (1997)	PBMC	ELISPOT	IL-4$^+$ cells ↑
Russell et al (1997)	PBMC	Cytokine secretion (PHA stimulation)	Th1 ↓
Matthiesen et al (1998)	PBMC	ELISPOT	Th1/Th2 →
Reinhard et al (1998)	PBMC	Flow cytometry	Th1 ↓, Th2 ↑
Saito et al (1999)	PBMC	Flow cytometry	Th1/Th2 ↓ (second and third trimester)
Tsuda et al (2001)	PBMC	Flow cytometry	Th2 ↑, Tc2 ↑
Zenclusen et al (2002)	PBMC	Flow cytometry	IL-12$^+$ granulocytes ↑
Bates et al (2002)	PBMC	Cytokine secretion	TNF-α ↓
Sacks et al (2003)	PBMC	Flow cytometry	Th1 ↓, Th2 →, IL-12$^+$ monocyte ↑
Krasnow et al (1996)	decidual tissue	ELISA	Th2 cytokines (IL-6, IL-10) ↑
Saito et al (1999)	decidual lymphocyte	Flow cytometry	Th1 ↓, Th2 ↑
Tsuda et al (2001)	decidual lymphocyte	Flow cytometry	Th2 ↓, Tc2 ↑
Michimata et al (2002)	decidual tissue	double immuno-fluorescence staining	Th2 ↓, Tc2 ↑ (decidua basalis)
von Rango et al (2003)	decidual tissue	RT-PCR	IFN-γ ↑ (decidua basalis) IL-4 ↑ (decidua basalis)

On the other hand, the Th1/Th2 cell ratios in the endometrium or decidua change dramatically during the menstrual cycle and pregnancy. For example, it has been reported that the Th1/Th2 ratio was 147.5 during the proliferative phase of the endometrium, 37.4 during the secretory phase and 1.3 in the early pregnancy decidua.[17] The same results have also been reported by Michimata et al.[13] They immunostained decidual sections for a specific Th2 and Tc2 marker, CHTH2, and the T-cell markers CD3 and CD8. Interestingly, the numbers and populations of Th2-type cells and Tc2-type cells were increased at the decidua basalis. There are at least two mechanism for increasing Type 2 cells at the implantation site. One mechanism is that chemoattractive factors produced by trophoblasts, endometrial grand cells and endometrial epithelial cells accumulate Type 2 cells at the implantation site. This assumption may be supported by the observation that Type 2 cells are seen around decidual blood vessels, endometrial gland cells, and extravillous trophoblasts, while few Type 2 cells are present around blood vessels in the decidua parietalis.[13] Drake et al[19] have reported that cytotrophoblasts can attract monocytes and CD56^{bright} NK cells by producing a chemokine, monocyte inflammatory protein (MIP)-1α. They have also reported that cytotrophoblast-conditioned medium contains a chemotactic factor for T cells, though they did not identify this substance.

Recently, Hirai et al[20] reported that CRTH2 is a second receptor for prostaglandin D_2 (PGD_2), and CRTH2 mediates the PGD_2-dependent cell migration of Th2 cells. Two PGD_2 synthase (PGDS) has been characterized as lipocalin-type or brain-type PGDS and hematopoietic PGDS (hPGDS), which is expressed in the placenta, Fallopian tube, lung and fetal liver. We have reported that hPGDS is expressed in not only maternal endometrial gland cells and endometrial epithelial cells, but also fetal trophoblasts[13], presumably resulting in the secretion of PGD_2 that functions as a chemoattractant for CRTH2-positive Type 2 cells. A chemokine receptor, CCR4 is preferentially expressed on Th2 cells, and its ligand, thymus and activation-regulated chemokine (TARC/CCL17) play important roles in the recruitment of Th2 cells. We reported that the percentage of $CCR4^+$ cells in $CD4^+$ T cells and $CD8^+$ T cells were significantly increased in human early pregnancy decidua compared with those in peripheral blood.[21] CRTH2 molecules were also expressed on $CCR4^+CD4^+$ T cells and $CCR4^+CD8^+$ T cells.[21] Unfortunately, the localization of decidual CCR4-positive T cells is unclear, because we did not succeed in immunostaining for CCR4. However, we found that trophoblasts, uterine epithelial cells and endometrial gland cells produce TARC, by immunohistochemical staining and the RT-PCR method. These findings suggest that TARC and PGD_2 play a role in accumulation of Th2 cells and Tc2 cells at the implantation site during early pregnancy.

As another mechanism for Type 2 cell accumulation at the implantation site, the immunological environment induces Th2- or Tc2-cell differentiation from naïve T cells. DCs are specialized antigen-presenting cells required for the priming and activation of T cells, and promote the differentiation of naïve $CD4^+$ T cells toward either the Th1 or Th2 phenotype. Peripheral blood DCs can be classified into $CD11c^+CD123^-$ myeloid DCs, which promote the Th1 response, and $CD11c^-CD123^+$ lymphoid DCs, which promote the Th2 response, by their surface markers. Unexpectedly, the population of lymphoid DC in the decidua was significantly lower than that in the peripheral blood.[22] However, the amount of IL-12 produced by total DC and myeloid DC in the decidua was significantly lower than that by peripheral myeloid DC. Importantly, naïve $CD4^+$ T cells primed with decidual myeloid DC led to a higher percentage of Th2 cells in comparison with that with peripheral myeloid DC.[22] These findings suggest that the DC in the decidua could regulate the Th1/Th2 balance to maintain a Th2- dominant state, leading to maintenance of pregnancy. High estrogen, PGE_2 and IL-10 inhibit production of IL-12, which is a key cytokine that induces Th1-type immunity. Since a high estrogen level, PGE_2 and IL-10 are present at the materno-fetal interface, these molecules might inhibit IL-12 production by decidual DCs, thereby shifting the Th1/Th2 balance to a Th2-dominant state at the interface. Progesterone has also been reported to induce the conversion of Th0 cells into Th2 cells.[23] A non-classical HLA class I gene, HLA-G is selectively expressed at high levels on the trophoblasts. Some have hypothesized that HLA-G may alter immunological events at this site via alterations in the cytokine expression pattern to a Th2-dominant status. HLA-G might have a role is successful embryonic implantation and/or subsequent pregnancy maintenance.

Thus, chemotactic factors and Th2-inducible factors may interact with each other within decidual tissues, resulting in Type 2-predominant immune conditions.

Th1/Th2 Balance in Sporadic Abortion or Unexplained Recurrent Spontaneous Abortion

Hill et al[24] first reported that Th1-type cytokine secretion was observed in women with recurrent pregnancy loss when peripheral blood mononuclear cells were activated by the trophoblast cell line Jeg-3. This finding is also supported by many reports (Table 3). Recently, Bates et al[25] reported the contrary results that Th2-type cytokine IL-4 and Tr-type cytokine IL-10 were significantly increased in women with recurrent spontaneous abortion (RSA). Zenclussen et al[26] reported that a lower percentage of IL-12 in peripheral blood lymphocytes, monocytes and granulocytes was observed in spontaneous abortion cases. Shimada et al[27] reported that the percentages of Th2 and Tc2 cells were significantly higher in RSA than those in control subjects.

Table 3. Peripheral blood Th1/Th2 balance in recurrent spontaneous abortion cases

Author	Materials and Methods	Results
Hill et al (1995); Raghupathy et al (1999); Makhseed et al (2001)	Cytokine secretion (mixed lymphocyte-placenta reaction)	Th1 ↑, Th2 ↓ Th1 ↑, Th2 ↓
Raghupathy et al (2000); Makhseed et al (2001)	Cytokine secretion (PHA stimulation) Cytokine secretion (PHA stimulation)	Th1 ↑, Th2 ↓ Th1 ↑, Th2 ↓
Jenking et al (2000); Makhseed et al (2000)	Cytokine in serum Cytokine in serum	Th1 ↑, Th2 ↓ Th1 ↑, Th2 ↓
Hayakawa et al (2000); Ho et al (2000) Ng et al (2002) Kwak-Kim et al (2003)	Flow cytometry Flow cytometry Flow cytometry Flow cytometry	Th1/Th2 ↑ Th2 ↓ or Th1 ↑ Th2 ↓, Tc2 →, Th1 → Th1/Th2 ↑, Tc1/Tc2 →
Palfi et al (1999) Rein et al (2002) Zenclussen et al (2002)	ELISPOT Flow cytometry Flow cytometry	Th1/Th2 → IFNγ $^{+}$PBMC → IL-12^{+} lymphocytes ↓ IL-12^{+} monocytes ↓
Bates et al (2002) Shimada et al (2003)	Cytokine secretion (PHA stimulation) Flow cytometry	Th1/Th2 ↓ Th1/Th2 ↓

These discrepancy might be explained as follows: (1) The etiology of RSA is varied, and includes maternal or paternal chromosomal aberrations, uterine anatomical abnormalities, endocrine disorders, infections and reproductive antiphospholipid syndrome. However, the etiology is undetermined in 40-60% of RSA cases: (2) Since the changes in Th1/Th2 ratios in peripheral blood are restricted within a narrow limit, a small number of cases showing abnormal Th1/Th2 ratio could affect the results: (3) Th1/Th2 ratio may be different between RSA with normal chromosomal content and RSA with abnormal chromosomal content. To clarify these points, we should examine Th1/Th2 balance in the deciduas, especially the decidua basalis, in RSA cases, especially RSA with normal chromosomal content. In cases of RSA with abnormal chromosomal content, Th2-type immunity might be present, because the major cause of abortion is fetal anomalies. Table 4 shows the decidual Th1/Th2 balance in RSA cases. Piccinni et al[28] first reported defective production of both LIF and Th2 cytokines by decidual T cells in unexplained RSA. Interestingly, production of Th1-type cytokines was similar between RSA and control subjects. However, they did not mention fetal chromosomal abnormality. We first reported that accumulation of Type 2 cells decreased in the decidua basalis in RSA with normal chromosomal content, although accumulation of Type 2 cells was observed in the decidua basalis in RSA with abnormal chromosomal content.[14] Interestingly, the populations of Type 2 cells, Th2 cells and Tc2 cells in the decidua parietalis were similar in normal pregnancy, RSA with normal chromosomal content and RSA with abnormal chromosomal content, suggesting that we should examine the immunological environment at the decidua basalis, and the Type 2-dominant immune environment at decidua basalis is important in maintaining pregnancy. To prove the hypothesis that a Th1-type response causes miscarriage clinically as documented in mice, the distribution and localization of Th1 cells and Tc1 cells should be examined. In our preliminary data, the number and population of Type 1 T cells and activated NK cells increased at the decidua basalis compare to those at the decidua parietalis in abortion cases. However, there were no differences between RSA with abnormal chromosome content and RSA with normal chromosome content, suggesting that increased Th1-type immunity

Table 4. Th1/Th2 balance in endometrium or decidua of recurrent spontaneous abortion cases

Author	Samples	Methods	Results
Lim et al (1999)	endometrium (RSA)	RT-PCR	Type 1 cytokines ↑
Wolf et al (2000)	endometrium (RSA)	RT-PCR	IL-1β ↓, IL-6 ↓
Piccinni et al (1998)	decidua (RSA)	T-cell clone	Th2 clones ↓, Th1 clones →
Vives et al (1999)	decidua (RSA)	RT-PCR	IFNγ mRNA ↑, TNFα RNA ↓
Zenclusen et al (2001)	decidua (SA)	Immunostaining	$CCR5^+$ cells (Th1 cells + monocytes) ↑
Ho et al (2001)	decidua (SA)	Flow cytometry	Type 2 (Th2 + Tc2) cells ↓, Type 1 cells →
Plevyak et al (2002)	decidua (SA)	Immunostaining	IL-10 ↓, IFNγ →
Zenclusen et al (2002)	decidua (SA)	Flow cytometry	$IL\text{-}12^+$ lymphocytes ↓
Michimata et al (2003)	decidua basalis (RSA)	double immuno-fluorescence staining	Th2 ↓, Tc2 ↓

might be a result of abortion. Evaluation of the ability of the immunophenotypes of endometrial leukocytes, especially Type 2 cells from patients with histories of recurrent abortion, is very important. Our results demonstrated that the numbers of Type 2 cells, $CD3^+$ T cells, $CD8^+$ T cells, NK cells, B cells and leukocytes in RSA in the mid-luteal phase of the endometrium were similar to those in controls.[29] Accumulation failure of Type 2 cells at the implantation site in RSA with normal chromosomal content might occur after implantation.

Regulatory T Cells in Pregnancy

The Th1/Th2 hypothesis has been further developed. T-cell subsets which produced the immunoregulatory cytokines IL-10 and TGF-β have been clarified. Th3 cells predominantly produce TGF-β, whereas Tr1 cells predominantly produce IL-10 (Fig. 2). Recently, it has been demonstrated that $CD4^+$ $CD25^+$ regulatory T cells play a central role in development of tolerance. The suppressive function of $CD4^+CD25^+$ regulatory T cells requires cell-to-cell contact, although they produce IL-10 and TGF-β. We first showed that the population of $CD4^+$ $CD25^+$ regulatory T cells in decidua from normal pregnancies is increased and they suppress the autologous proliferation of $CD4^+$ $CD25^-$ T cells.[30] Heikkinen et al[31] and Somerset et al[32] also reported the same results. In contrast, the population of decidual $CD4^+$ $CD25^+$ regulatory T cells was significantly lower in specimens from spontaneous abortion compared to those from induced abortion.[30] These results suggest that decidual $CD4^+$ $CD25^+$ regulatory T cells might inhibit the maternal immune response and therefore might contribute to the maintenance of pregnancy. In murine pregnancy, $CD4^+$ $CD25^+$ regulatory T cells mediate maternal tolerance to the fetus.[33] The absence of regulatory T cells led to a failure of gestation due to immunological rejection of the fetus. Furthermore, regulatory T cells play very important roles for not only successful pregnancy, but also implantation.[34] The anti-CD25 treatment resulted in a decrease in the percentage of successful implantation.[34] Further studies are needed to clarify the localization of $CD4^+$ $CD25^+$ regulatory T cells, especially at the decidua basalis, in RSA with normal chromosomal content. The distribution of Th3 and Tr1 cells in the decidua has not been published. We should clarify these points. Analysis of cytokines and differentiation of antigen expression in human NK cells disproves the NK1-NK2 hypothesis. NK cells can be classified into NK1 cells and NK2 cells by their cytokine production. It has not been reported whether NK1 cells and NK2 cells are present in the decidua, or whether NK2 cells are dominant in the decidua. Loza et al[12] proposed that the cytokine environment regulates NK2→NK0→NK1

Table 5. Th1/Th2 balance in multiple implantation failure cases

Author	Samples	Methods	Results
Ng et al (2002)	Peripheral blood	Flow cytometry	$TNF\alpha^+CD4^+T \uparrow$, $IL\text{-}10^+CD8^+ T \downarrow$ $IFN\gamma^+\text{-}T \rightarrow$, $IL\text{-}4^+\text{-}T \rightarrow$
Kwak-Kim et al (2003)	Peripheral blood	Flow cytometry	Th1/Th2 ↑, Tc1/Tc2 →

development progression, with IL-12 needed for terminal differentiation and IL-4 delaying this process. The cytokine environment at the materno-fetal interface suggests that NK2 might be a dominant state in early pregnancy decidua.

Th1/Th2 Balance at Implantation Stage

In non-pregnant women with a normal estrous cycle, T cells within the uterine endometrium show a very strong Th1 bias during the proliferative phase (Th1/Th2 ratio: 147.5±96.7)[17]. The ratio of Th1:Th2 is reduced during the secretory phase (Th1/Th2 ratio: 37.7±21.3). The Th1/Th2 ratio in secretory phase endometrium is significantly higher than that in peripheral blood (10.2±3.5), suggesting a more Th1-like cytokine bias in the uterine tissue at the time of implantation. Hunt et al[35] reported that the level of Th1-type cytokine TNF-α mRNA is very high during the implantation window. These data suggest that a transient local Th1-type environment, typified by TNF-α produced by uterine endometrial cells and leukocytes, may be induced at the time of implantation. IFN-γ, while being abortifacient if injected in high doses, especially in synergy with TNF-α, is required at lower doses for proper activation of uterine NK-cell function. These activated uterine NK cells have some roles in angiogenesis and placentation in mice[36] Chaouat et al[37] examined expression of IL-12 and IL-18, and distribution of NK cells in mid-luteal phase endometrium in infertile women. They pointed out that these patients can be classified to two groups, cytokine and NK cell excess, and cytokine depletion. These findings suggest that moderate Type 1 cytokine stimulation might be essential, but excessive Type 1 cytokine stimulation might induce implantation failure. For prevention of this excessive stimulation, regulatory cytokines and Type 2 cytokines might regulate the endometrial immune system. The failure to synchronize the component processes involved in these interactions results in a failure of implantation.

According to mouse models, maternal T cells in the uterus play an important role in implantation. Recent data suggest that excessive Th1-type immunity in peripheral blood might be present in multiple implantation failure (Table 5). However, Th1/Th2 balance in the mid-luteal phase endometrium of multiple implantation failure cases has not been clarified.

LIF is known as an essential cytokine in implantation in mice. Also, diminished secretion of LIF is associated with recurrent pregnancy loss. In humans, LIF is secreted from the endometrial epithelium, $CD16^-CD56^{bright}$ NK cells and Th2 cells. Therefore, maternal immunocompetent cells could assist the implantation process by LIF secretion. Since the migration of white blood cells is promoted by chemotactic factors, it would appear that various chemotactic factors are present in endometrium during the implantation stage. It was reported that seminal 'priming' is important for successful mammalian pregnancy.[38] Interestingly, seminal plasma contains the chemotactic factor MIP-1α for $CD16^-CD56^{bright}$ cells, and lipocalin-type PGDS, which produces PGD_2. PGD_2 is known as a chemoattractive factor for Th2 cells, and cyclic changes in PGD_2 release are found only in endometrium with increased rates during menstruation and mid-luteal phase. So, seminal priming might increase the population of Th2 cells and $CD16^-CD56^{bright}$ NK cells in the endometrium. Indeed, Tremellen et al[39] reported that seminal priming increased the pregnancy rates during IVF-ET. Seminal plasma also contains the immunoregulatory cytokine TGF-β. TGF-β initiates the inflammatory process by stimulating the synthesis of pro-inflammatory cytokines and chemokines in uterine tissues. TGF-β is likely

to be the key cytokine in skewing the immune response against a Type 1 bias. Although implantation appears to be achieved by the cross-talk between the regulation of chemokines introduced via seminal fluid, sex hormones and embryonic-derived factors, further studies on the role of cytokines, especially the Th1/Th2/Th3 balance, in implantation are urgently needed.

Summary and Conclusions

The cytokine profile in the endometrium clearly changes during implantation and pregnancy. These dramatic and synchronized changes are present at the local implantation site. We could not detect these local immunological changes by examination of peripheral blood. We should focus on the localized immune system rather than the systemic immune system. There is a bias in the ratio of Th1:Th2 cytokines towards Th2-type cytokines at the materno-fetal interface. The high Th1/Th2 ratio (147.5±96.7) in the proliferative phase of the endometrium decreases to 1.3±0.5 in the early pregnancy decidua, suggesting that a Th2-predominant state is present in the pregnant uterus. However, an almost equal number of Th1 cells is also present in the pregnant uterus. We should understand the role of Th1 cells in implantation and placentation. At implantation, the cytokine balance is rather Type 1-dominant. However, during placental formation there is a clear bias towards Th2-type cytokines.[40] Although these processes have not been clarified, recent studies suggest maternal T cells play a central role in implantation and Th2 and Tc2 cells accumulate at the implantation site. A better understanding of these sequential events could improve clinicians' ability to treat disorders related to these processes, including infertility and early pregnancy loss.

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CHAPTER 5

The Regulation of Human Trophoblast Apoptosis and Survival during Pregnancy

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Abstract

Apoptosis occurs in the villous trophoblast of normal placentas throughout pregnancy, but with higher frequency near term in comparison to the first trimester. In pregnancies complicated by preeclampsia or intrauterine growth restriction (IUGR), a greater incidence of villous and extravillous trophoblast apoptosis has been observed, suggesting that the deregulation of trophoblast apoptosis may contribute to pathological conditions. Apoptosis may be initiated by one of two known pathways, the mitochondrial (instrinsic) pathway and the death receptor-mediated (extrinsic) pathway, or in response to exogenous stimuli such as cytokines or hypoxia. The central executioners of apoptosis are the caspase family of cysteine proteases, which cleave numerous vital cellular proteins to affect the apoptotic cascade. By controlling caspase activation, several endogenous inhibitors, including FLIP, IAPs and anti-apoptotic Bcl-2 family members prevent further propagation of the death signal. This review focuses on the molecular mechanisms by which normal trophoblast apoptosis occurs and how it is regulated to avoid excessive trophoblast apoptosis and pregnancy complications.

Introduction

Apoptosis, or programmed cell death, is an active process by which superfluous or dysfunctional cells are eliminated in order to maintain normal tissue function. During pregnancy, apoptosis plays an important role in several aspects of placental biology including interstitial and endovascular invasion by extravillous trophoblasts,[1,2] maternal immune tolerance[1,3,4] and trophoblast differentiation and turnover.[4-6] Apoptosis has been detected in the placenta throughout normal pregnancy, however, a higher frequency occurs in the villous trophoblast of third trimester placentas than that of first trimester placentas.[7,8] A greater incidence of villous[9-12] as well as extravillous[13,14] trophoblast apoptosis has been observed in placentas from pregnancies complicated by preeclampsia or intrauterine growth restriction (IUGR), suggesting that alterations in the regulation of trophoblast apoptosis may contribute to pathological conditions.

The main objective of this review is to discuss the molecular mechanisms by which trophoblast cell apoptosis is regulated during normal pregnancy and the alterations found in pregnancy complications.

Death Receptor-Mediated Apoptosis

In response to different stimuli, apoptosis can be initiated by one of two known pathways; the mitochondrial, or instrinsic pathway and the death receptor-mediated, or extrinsic pathway. If executed by the extrinsic pathway, apoptosis is initiated by members of the Tumor Necrosis Factor-Receptor (TNF-R) family, which have a C-terminal region of approximately

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80 amino acids known as the death domain (DD) in common.[15] To date, eight members of this family have been identified and include Fas (CD95/APO-1), TNF-R1 (CD120a), DR-3 (APO-3/WSL-1/TRAMP/LARD), TRAIL-R1 (DR-4), TRAIL-R2 (DR-5/TRICK2), DR-6, EDAR and NGFR.[16] Among the death receptors, Fas, TNF-R1 and TRAIL-R1/TRAIL-R2 are the most widely studied and best characterized. The interaction between each membrane-bound death receptor and corresponding ligand(s) results in the activation of preassociated death receptor trimers[17,18] and the recruitment of several adaptor proteins to the inner cell membrane,[19] the point at which the TNF death receptor pathways converge.

Along with several others, we have shown that both villous[20-24] and extravillous trophoblast cells[1,20,25,26] express Fas and/or Fas Ligand (FasL), but are resistant to Fas-mediated apoptosis under normal conditions.[22,27,28] More recently, we also demonstrated that first trimester trophoblast cells secrete functional FasL to induce apoptosis in Fas-bearing immune cells and avoid maternal immune surveillance and rejection.[3] The expression of TNF-R1 has also been localized to villous[29-31] as well as extravillous trophoblast,[29] but in contrast to Fas, we and others have shown that trophoblast cells are sensitive to TNF-α, the ligand for TNF-R1.[22,27,30,31] Even though trophoblast cells do not express TRAIL, it was previously demonstrated that these cells do express TRAIL-R1 and TRAIL-R2 and are resistant to recombinant TRAIL.[31,32] Since these studies were performed with either trophoblast-derived choriocarcinoma cell lines[32] or term trophoblast cells,[31] however, whether normal first trimester trophoblast cells exhibit a similar expression pattern is unknown. In addition, DR-3 and DR-6 have also been shown to be expressed by term trophoblast cells,[31] while trophoblast expression of the DR-3 ligand, TLA-1,[33] has not been reported and a ligand for DR-6 has yet to be identified. Therefore, whether or not DR-3 and DR-6 function in trophoblast cells remains to be determined.

Interestingly, trophoblast cells express decoy receptors as well as several non-death domain containing TNF receptors such as TNF-R2, LTβR and HVEM and their respective ligands TNF-α, LT-α/LT-β and LIGHT,[31,34] which can also bind multiple TNF family members. Although capable of inducing apoptosis in other cell types, it was previously shown that at least TNF-R2 does not participate in TNF-α-induced trophoblast cell apoptosis and that apoptosis induced by TNF-α is mediated almost entirely by TNF-R1.[30] This may be explained by studies illustrating that signals transmitted by TNF-R2, as well as the prototypical death receptors, TNF-R1, TRAIL-R2 and Fas may also promote growth in certain cells through the activation of nuclear factor-kappaB (NF-κB) and mitogen-activated kinases such as c-jun N-terminal kinase (JNK).[35-37] In support of this, we recently demonstrated that first trimester trophoblast cells can actually proliferate upon Fas stimulation.[28] Altogether, these studies suggest that trophoblast cells possess multiple death receptors, including those that lack death domains, but how these pathways function simultaneously to regulate trophoblast cell apoptosis and/or survival throughout pregnancy is only beginning to be understood.

The Extrinsic Pathway

In order to transduce apoptotic signals, TNF death receptors have intracellular death domains (DD), which mediate protein-protein interactions with other death domain-containing adaptor proteins such as the Fas-associated death domain (FADD; MORT1) and TNRF-associated death domain (TRADD).[38] Once FADD binds to the cytoplamic tail of the death receptor either directly or indirectly through TRADD in the case of TNF-R1,[39] it recruits other cellular proteins, including procaspase-8 and procaspase-10[40,41] via death effector domains (DED) to form the death-inducing signaling complex (DISC)[19] (Fig. 1). Caspases are family of cysteine proteases that can be subdivided into two groups; the initiator caspases, which initiate apoptosis by activating the second group, the downstream effector caspases. Analogous to other proteases, caspases are synthesized as inactive precursors termed procaspases that exist in this pro-form until activated.[42] Once the DISC is assembled, 'initiator' caspase-8 and caspase-10 become activated,[43] but the mechanism by which this occurs is still unclear. According to the induced proximity model, high local concentrations of procaspase-8 or

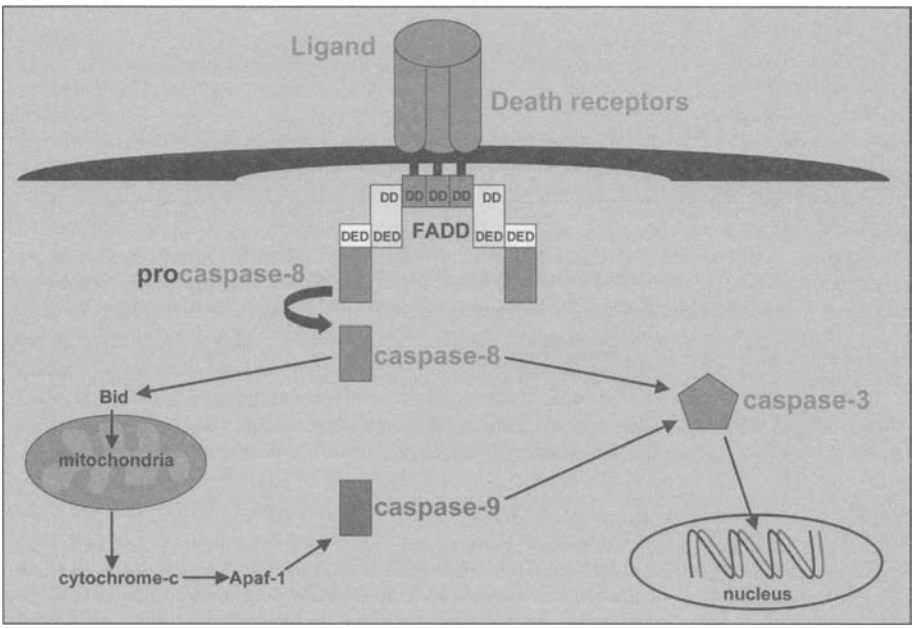

Figure 1. The extrinsic and intrinsic apoptotic pathways.

procaspase-10 are auto-catalytically activated in a two-step cleavage process while in close proximity to the DISC, resulting in the formation of active caspase-8 or caspase-10 heterotetramers.[44] Recent data, however, suggests that caspase-8 activation arises as a result of dimerization rather than proteolytic processing as such and that activation of caspase-8 can occur independently of the DISC.[45] Nevertheless, caspase-8 and caspase-10, once activated, initiate the caspase cascade by activating 'effector' caspase-3, caspase-6 and caspase-7.[46]

The Intrinsic Pathway

Unlike the extrinsic pathway, which depends on death receptor signaling, in the intrinsic pathway, the apoptotic signal is initiated by or directed to the mitochondria. The extrinsic and intrinsic pathways are not necessarily independent, however, since crosstalk can occur between the two in certain cell types and the mitochondrial pathway may amplify signals triggered by death receptors. Besides activating effector caspases, caspase-8 can also cleave Bid, a pro-apoptotic Bcl-2 family member, resulting in the translocation of truncated Bid (tBid) to the mitochondria and the activation of the intrinsic pathway[47] (Fig. 1). Consequently, other pro-apoptotic Bcl-2 family members such as Bax and Bak increase the permeability of the mitochondrial membrane to cytochrome-c release by a highly controversial mechanism.[31,48,49] As a result, cytochrome c binds the adaptor protein, apoptotic protease activating factor (APAF-1), which subsequently binds and activates 'initiator' caspase-9, forming a macromolecular complex called an apoptosome.[50] In turn, caspase-9 activates caspase-3 and caspase-7, the point at which the mitochondrial and death receptor pathways overlap.[46] As 'effector' caspases, caspase-3, caspase-6 and caspase-7 cleave a variety of vital cellular proteins, including DNA repair enzymes, nuclear lamins and cytosketetal proteins,[51] which explains the characteristic features of apoptosis such as nuclear condensation, membrane blebbing and cell shrinkage. In addition, inhibitor of caspase-activated deoxyribonuclease (ICAD) is also cleaved by effector caspases, releasing caspase-activated deoxyribonuclease (CAD) to non-specifically cut the genomic DNA into approximately 200 base pair fragments,[52] eventually ending in the demise of a cell.

The Apoptotic Cascade in Trophoblast Cells

Numerous studies have identified nuclear condensation, membrane blebbing, DNA fragmentation and/or other morphological changes consistent with apoptosis in villous trophoblast from uncomplicated pregnancies,[5,7,8,53] suggesting that placental apoptosis occurs during normal pregnancy. Villous trophoblast consists of two subpopulations; proliferating mononuclear cytotrophoblast cells, which continuously fuse to form the multi-nucleated syncytiotrophoblast, the layer of trophoblast, which is in direct contact with the maternal circulation.[54] Since apoptosis has been observed in normal villous trophoblast, it was postulated that caspases may be involved in trophoblast differentiation and cell turnover. Indeed, Huppertz et al previously demonstrated that first and third trimester cytotrophoblast cells only express the pro-forms of caspase-3, caspase-6 and caspase-7, whereas the active forms of caspase-3 and caspase-6 are observed in syncytiotrophoblasts following syncytial fusion. This was further supported by the detection of poly (ADP-ribose) polymerase (PARP) and nuclear lamin B, two effector caspase substrates, only in the nuclei of villous cytotrophoblasts and the degradation product of PARP in syncytiotrophoblasts.[4,55] Using the M30 antibody, which recognizes a neo-epitope that is exposed only after effector caspase-mediated cleavage of the cytoskeleton-associated protein, cytokertain-18, it was recently shown that the majority of M30-postive cells in villous tissue are the syncytiotrophoblasts.[56,57] In contrast, Yusuf et al demonstrated that not only do third trimester cytotrophoblast cells have effector caspase activity, but that the activity of caspase-3 and caspase-6, as well as 'initiator' caspase-9, is greater in cytotrophoblasts than in syncytiotrophoblasts,[58] which may explain why cytotrophoblast cells from third trimester placentas also exhibit a higher level of apoptosis than syncytiotrophoblasts in culture.[59] Both groups agree, however, that caspase-8 activity is more prevalent in first[55] and third trimester cytotrophoblasts[58] than in syncytiotrophoblasts. In support of this, Huppertz's group also reported the loss of fodrin, a cytoskeletal protein that is initially cleaved by active caspase-8, in villous cytotrophoblast.[55] Therefore, it was hypothesized that caspase-8 activity may be required for the fusion of cytotrophoblast cells with the syncytiotrophoblast layer. Using antisense oligonucleotides and peptide inhibitors to inhibit caspase-8 expression and activity, respectively, the same group recently demonstrated that the inhibition of caspase-8 reduces syncytial fusion, thereby preventing progression of the apoptotic cascade in syncytiotrophoblast and resulting in the accumulation of mononucleated cytotrophoblast.[60] This confirmed that caspase activation is initiated in villous cytotrophoblast cells, which in turn, promotes cytotrophoblast fusion with syncytiotrophoblast and formation of the syncytial layer. However, an important question that was raised from these studies is how the trophoblast survives in spite of caspase activation. More specifically, (i) what prevents apoptotic signaling events downstream of caspase-8 activation in cytotrophoblast cells, (ii) which other components of the apoptotic cascade are involved in this process and (iii) how trophoblast apoptosis is regulated in parallel with syncytial fusion during pregnancy.

Endogenous Regulators of Trophoblast Apoptosis

Flice-Like Inhibitory Protein (FLIP)

Each step of the apoptotic cascade is tightly controlled by several endogenous inhibitors, which prevent further propagation of the death signal either at the "initiator" or "effector" level (Fig. 2). By precluding caspase-8 recruitment to the DISC, Flice-like inhibitory protein (FLIP) inhibits apoptosis triggered by death receptors.[61] In support of this, we previously demonstrated that interleukin-10 (IL-10)-induced FLIP expression and activation protects first trimester trophoblast cells from Fas-mediated apoptosis.[27] Moreover, FLIP has also been shown to induce cell proliferation by activating survival pathways such as NF-κB.[62] Although multiple splice variants of FLIP exist, only FLIP long ($FLIP_L$) and FLIP short ($FLIP_S$) have been detected in the placenta thus far.[27,61] $FLIP_L$ and $FLIP_S$ each contain two DEDs, but $FLIP_L$ also has a domain that is homologous to the catalytic domain of caspase-8. Unlike caspase-8,

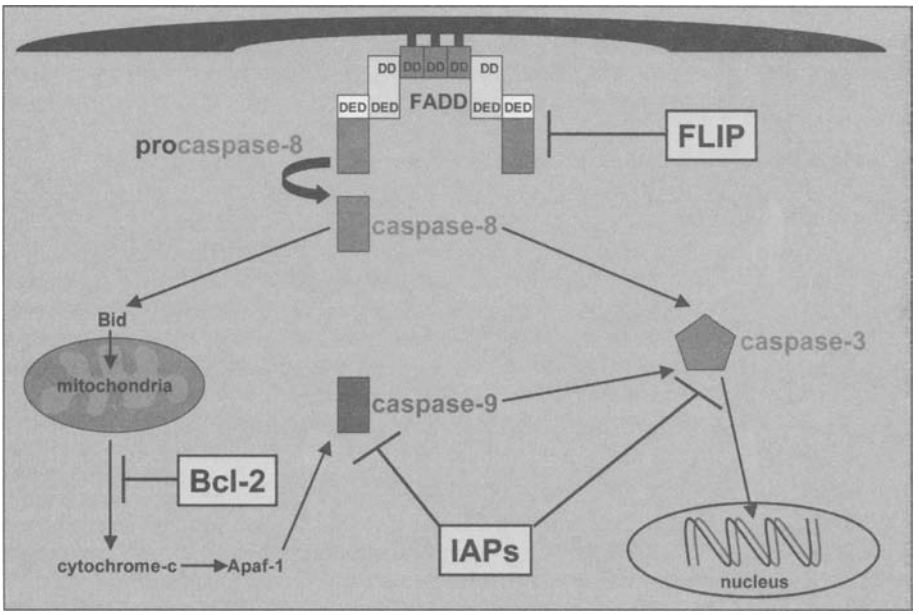

Figure 2. Intracellular regulation of the apoptotic cascade.

however, $FLIP_L$ is catalytically inactive and lacks the structural components to activate downstream caspases.[63] It was originally thought that $FLIP_L$ and $FLIP_S$ compete with caspase-8 for binding to FADD since both can also be recruited to the DISC via DEDs. In the presence of $FLIP_L$, however, caspase-8 is not completely precluded from the DISC, whereas $FLIP_S$ prevents all caspase-8 processing. The authors speculated that the initial caspase-8 cleavage step occurs auto-catalytically and requires the presence of a caspase domain. Even though the caspase domain of $FLIP_L$ is inactive, it may be sufficient to cleave caspase-8. Since $FLIP_S$ lacks a caspase domain, this might explain why $FLIP_S$ prevents all caspase-8 processing, while caspase domain-containing $FLIP_L$ is only able to inhibit the second caspase-8 cleavage step.[64] More recently, however, it was shown that the long form of FLIP may actually promote caspase-8 activation by dimerizing with caspase-8,[65,66] which suggests that the activation of caspase-8 can occur in the absence of proteolytic processing. This was confirmed by recent studies demonstrating that dimerization is both necessary and sufficient for caspase-8 activation, whereas caspase-8 processing is neither required nor sufficient for catalytic activity.[67,68] Therefore, whether or not the caspase cascade is initiated following death receptor activation may depend on the stoichiometry of $FLIP_L$, $FLIP_S$ and caspase-8 at the DISC.

Although $FLIP_L$ and $FLIP_S$ expression was identified in the placenta several years ago,[61] relatively little is known about their role in trophoblast cells. We recently demonstrated that FLIP expression is important for protecting trophoblast cells from Fas-mediated apoptosis.[27] Further studies are necessary to determine how FLIP functions in the placenta, whether there are any differences between $FLIP_L$, $FLIP_S$ or other isoforms and how the expression of FLIP is regulated in trophoblast cells during pregnancy.

The Bcl-2 Family

In contrast to FLIP, which inhibits apoptosis triggered by death receptors, Bcl-2 family members can differentially modulate death signals either directed towards or initiated by the mitochondrial pathway. Named after the founding member of the family, which was isolated as a gene involved in B-cell lymphoma (Bcl),[69] the Bcl-2 family is comprised of three functional

groups, which are characterized by varying numbers of Bcl-2 homology (BH) domains. Members of the first group, such as Bcl-2 and Bcl-x_L, contain four BH domains (BH1-BH4) and inhibit apoptosis, whereas Bax and Bak, which are part of the second group, are pro-apoptotic and lack a N-terminal BH4 domain. The BH3-only proteins, Bid and Bik, are members of the third group and promote apoptosis by either activating pro-apoptotic members such as Bax and Bak or binding anti-apoptotic Bcl-2 family members to inhibit their function. The mechanism by which this occurs, however, is not completely understood.[70] Nevertheless, Bax and Bak increase the permeability of the mitochondrial membrane to cytochrome-c release, while Bcl-2 and Bcl-x_L inhibit the release of cytochrome-c and other pro-apoptotic factors from mitochondria, thereby preventing further propagation of the apoptotic signal.[48,71] Thus, the outcome may depend on the relative abundance of pro vs. anti-apoptotic family members in the cell.

Initially, it was unclear as to whether Bcl-2 was involved in apoptosis initiated by death receptors since studies demonstrating that the overexpression of Bcl-2 inhibited Fas-mediated apoptosis were equally opposed by studies indicating that it does not. This was subsequently resolved with the identification of two different cell types, which utilize one of two Fas signaling pathways.[72] In type I cells, enough active caspase-8 is produced to directly activate caspase-3, whereas only a small amount of caspase-8 is generated in type II cells. Although not sufficient to activate caspase-3, caspase-8 is able to cleave Bid in type II cells, resulting in the activation of the intrinsic pathway. As a consequence, type I cells undergo death receptor-mediated apoptosis without mitochondrial support, whereas type II cells require the release of cytochrome-c from mitochondria in order to induce apoptosis initiated by Fas or other death receptors. Therefore, Bcl-2 or Bcl-x_L can only inhibit death receptor-mediated apoptosis in type II cells. Interestingly, the expression of Bcl-2 does not completely protect cytotrophoblast cells from TNF-α-induced apoptosis[73] and villous cytotrophoblasts exhibit high levels of caspase-8 activity,[55,58] which suggests that trophoblast cells are a type I cell and do not require the mitochondrial pathway to undergo death receptor-mediated apoptosis. Moreover, it also implies that other regulators outside of the mitochondria exist to prevent apoptosis from occurring in the trophoblast. The type and strength of stimulus, as well as the local microenvironment at the maternal-fetal interface, however, may also influence whether apoptosis is executed independently of the mitochondrial pathway or the use of the intrinsic pathway is necessary to amplify the death receptor signal.[74,75]

Although there is some discrepancy as to whether Bcl-2 expression is lower in syncytiotrophoblasts from first trimester placentas than in third trimester syncytiotrophoblasts,[76-78] several groups have shown that syncytiotrophoblasts express Bcl-2 at higher levels than villous cytotrophoblasts from both trimesters of pregnancy.[4,76,77,79-82] Similarly, the expression of Mcl-1, another anti-apoptotic Bcl-2-related family member, is more prominent in synciotrophoblasts compared to cytotrophoblast cells.[4,80] Therefore, Bcl-2 and Mcl-l appear to be involved in preventing syncytiotrophoblast apoptosis following syncytial fusion. However, there is also evidence indicating that Bcl-2 and Mcl-1 do not completely protect the syncytium from apoptosis,[7,8] which may be explained by studies demonstrating that syncytiotrophoblasts also express the pro-apoptotic Bcl-2 family members, Bax[78] and Bak.[81] This suggests that the ratio of anti to pro-apoptotic Bcl-2 family members may determine whether trophoblast cell death or survival prevails.[78,79]

Inhibitors of Apoptosis (IAPs)

Unlike FLIP and the anti-apoptotic members of the Bcl-2 family, inhibitors of apoptosis (IAPs) are unique in that they are capable of inhibiting both the mitochondrial and death receptor-mediated pathways. To date, eight human IAPs have been identified and include X-linked inhibitor of apoptosis (XIAP;MIHA/ILP-1), ILP-2 (Ts-IAP), c-IAP1 (HIAP2/MIHB), c-IAP2 (HIAP1/MIHC), neuronal apoptosis inhibitory protein (NIAP), survivin (TIAP), Livin (KIAP/ML-IAP), and Apollon (Bruce). IAP family members are characterized by varying

numbers of baculoviral IAP repeat (BIR) domains and with the exception of NIAP, survivin and Apollon, also contain a C-terminal RING (really interesting new gene)-zinc finger domain.[83] The RING domain was previously shown to have E3 ubiquitin ligase activity, which enables IAPs to ubiquitinate and degrade themselves or other interacting proteins following certain apoptotic stimuli.[84] In addition, c-IAP1 and c-IAP2 also contain a caspase-recruitment domain (CARD),[85] the function of which in these proteins is still unknown.[86]

XIAP, c-IAP1, c-IAP2, NAIP, survivin and livin all have been shown to be expressed in villous trophoblasts and except for c-IAP1, in extravillous trophoblast cells as well.[24,87-89] The expression of Apollon has not yet been studied in the trophoblast, however, and the function of only XIAP and survivin have been evaluated in cytotrophoblast cells.[28,89] Although survivin consists of only one BIR domain, it is sufficient to inhibit caspase-9 during mitosis,[90] and possibly caspase-3 and caspase-7,[91] but this is still in debate.[92] to associate with microtubules of the mitotic spindle and spindle poles, centrosomes and the kinetochores of metaphase chromosomes,[93] suggesting that survivin may be involved in both the control of apoptosis and regulation of cell division during mitosis. Interestingly, the expression of survivin is regulated in a cell-cycle-dependent manner and peaks at the G_2-M phase of the cell cycle.[94] Two splice variants of survivin have been identified, one lacking exon 3 (survivin-deltaEx3) and another retaining a part of intron 2 (survivin-2B),[95] but neither variant has been detected in the placenta so far.[89,96] The expression of survivin was localized to villous cytotrophoblasts of first trimester placentas,[87] which was confirmed by recent studies identifying positive survivin immunoreactivity in both first and third trimester villous and extravillous cytotrophoblasts, with weaker staining observed in the syncytiotrophoblast layer.[88,89] Using antisense oligonucleotides to inhibit survivin expression, Shiozaki et al also demonstrated that the inhibition of survivin induced apoptosis in a trophoblast cell line.[89]

Among the IAPs, XIAP is the most potent and versatile member of the family.[97] XIAP contains three tandem BIR domains, which have been shown to differentially inhibit initiator and effector caspases.[98] While the BIR1-BIR2 linker together with the BIR2 domain of XIAP has been shown to prevent caspase-3 and caspase-7 activation,[99] the caspase-9 inhibitory activity of XIAP has been localized to the BIR3-RING domain.[100] Besides inhibiting caspase function, XIAP has also been suggested to be involved in other cellular processes, including receptor-mediated signal transduction.[101] In response to certain apoptotic stimuli, XIAP was previously shown to be cleaved into two distinct fragments, a N-terminal fragment containing BIR1-2 and a second fragment containing BIR3-RING. The BIR1-2 fragment has diminished ability to inhibit caspase-3 and may be susceptible to further caspase-mediated degradation, whereas the BIR3-RING fragment is more stable and retains the ability to inhibit caspase-9, but is unable to suppress Fas-induced apoptosis.[102] XIAP expression was previously identified in villous cytotrophoblasts and syncytiotrophoblasts of first trimester placentas and shown to significantly decrease in third trimester placentas, which correlated with an increase in placental apoptosis.[24] However, only the expression of the full-length form of XIAP was characterized in placental tissue samples. More recently, we demonstrated that the active form of XIAP is primarily expressed in first trimester placentas, whereas the predominant form detected in term placentas is the inactive fragment of XIAP. Primary trophoblast cells isolated from first trimester placentas were shown to express only the active form of XIAP, while the inactive fragment of XIAP could not be detected, confirming that the trophoblast was the placental cell type responsible for the expression of XIAP. In addition, we demonstrated that XIAP inactivation renders normally resistant first trimester trophoblast cells sensitive to Fas-mediated apoptosis, evidenced by a decrease in trophoblast cell viability and increase in the activation of caspase-8, caspase-9 and caspase-3. Although some caspase-8 activation was observed in untreated first trimester trophoblast cells, caspase-9 and caspase-3 activation could not be detected and the cells did not undergo apoptosis in response to Fas stimulation.[28] As previously mentioned, cytotrophoblasts exhibit high levels of caspase-8 activity,[55,58] which is important for cytotrophoblast fusion and formation of the syncytial layer.[60] Therefore, we hypothesize

that XIAP protects trophoblast cells from the pro-apoptotic effect of caspase-8 activity by inhibiting caspase-9 and caspase-3 activation, thereby promoting trophoblast differentiation. If the expression of XIAP decreases, as in villous cytotrophoblasts and syncytiotrophoblasts of third trimester placentas,[24] the protective effect is removed and an increase in villous trophoblast apoptosis is observed.[7,8] Future studies are needed to determine whether other IAP family members besides XIAP and survivin can inhibit trophoblast apoptosis and how IAPs are regulated in trophoblast cells.

Exogenous Regulation of Trophoblast Apoptosis

The term, programmed cell death, is typically used to describe apoptosis induced during normal tissue turnover. However, apoptosis may also be in response to exogenous stimuli such as cytokines, which are not part of the developmental program of a cell. Numerous studies from both humans and murine models support the notion that the predominance of anti-inflammatory over pro-inflammatory cytokines at the maternal-fetal interface is essential for successful pregnancy.[103,104] This cytokine profile not only promotes immune protection, but it may also regulate trophoblast survival.[105] One of the possible mechanisms by which cytokines affect trophoblast survival may be by regulating the expression and/or function of components of the apoptotic cascade. It was previously demonstrated by our laboratory and others' that the pro-inflammatory cytokines, TNF-α and interferon-gamma (IFN-γ), induce trophoblast apoptosis by upregulating Fas expression[27] and increasing caspase-3 activity in first trimeter cytotrophoblast cells.[27,29] In contrast, treatment with Interleukin-10 (IL-10), an anti-inflammatory cytokine, inhibits the apoptotic effect of TNF-α and IFN-γ treatment on trophoblast cells. On the one hand, IL-10 treatment increases FasL expression in trophoblast cells, thereby promoting trophoblast immune protection, but on the other, it also increases the expression of $FLIP_L$,[27] which may protect against the autocrine or paracrine effects of FasL expression. Similarly, exposure to epidermal growth factor (EGF) has also been shown to block TNF-α/IFN-γ-induced apoptosis in cytotrophoblasts and syncytiotrophoblasts from third trimester placentas, but the mechanism by which this occurs is still unclear.[106] It is known, however, that treatment with EGF, as well as TNF-α or IFN-γ, does not affect the expression of Bcl-2[73] and that EGF protects from ceramide and acid sphingomyelinase (A Smase)-induced trophoblast apoptosis by decreasing the level of ceramide, a lipid-derived second messenger in the sphingomyelin pathway that is associated with TNF-α and Fas-mediated apoptosis in trophoblast cells.[107] In addition, basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1) and platelet-derived growth factor (PDGF) have all been shown to provide partial protection against TNF-α and IFN-γ treatment, whereas vascular endothelial growth factor (VEGF) and placenta growth factor (PLGF) do not protect trophoblast cells from TNF-α/IFN-γ-induced apoptosis, but the mechanisms of action remain to be elucidated.[108,109]

Additional studies by Desai et al also demonstrated that PlGF can induce the activation of survival pathways such as c-Jun-N terminal kinase (JNK), stress-activated protein kinase (SAPK) and p38 kinase, but not the extracellular signal regulated kinase-1 and -2 (ERK-1 and -2) pathways in trophoblast cells in response to growth factor withdrawal.[109] Altogether, this network of different cytokines and growth factors may promote or inhibit trophoblast apoptosis by influencing the expression of either pro or anti-apoptotic factors in the apoptotic cascade or by activating survival pathways.

Trophoblast Apoptosis and Complicated Pregnancies

A greater incidence of villous[9-12] as well as extravillous[13,14] trophoblast apoptosis has been detected in placentas from pregnancies complicated by preeclampsia or IUGR. This increase in trophoblast cell death might explain the insufficient trophoblast invasion often found in abnormal pregnancies. If increased trophoblast apoptosis occurs early in pregnancy, it may limit interstitial and endovascular invasion by extravillous trophoblasts.[13,14,110] However, a recent study by Kadyrov et al, suggests that the reduced trophoblast invasion observed in

preeclampsia is not associated with higher rates of apoptosis since a decrease in interstitial trophoblast apoptosis was observed in preeclamptic placentas compared to the normal controls.[111] This could be the result of when the samples were analyzed. The samples evaluated in this study were obtained from patients with active disease and, therefore, the presence of apoptotic cells may have preceded the time of analysis. Nevertheless, the original studies also demonstrated that the apoptotic extravillous cytotrophoblast cells in preeclamptic samples were negative for Bcl-2 expression.[13,14] Similarly, the expression of Bcl-2 was also shown to be less abundant in syncytiotrophoblasts from severe preeclamptic and IUGR placentas,[12] whereas earlier studies found no difference in Bcl-2 expression, as well as in the expression of Bcl-x_L, Bax and Bak in placental villi from complicated and normal pregnancies,[9,112] Levy et al, however, did find that the expression of p53, a tumor suppressor that activates pro-apoptotic Bcl-2 family members such as Bax in response to DNA damage,[113] was upregulated in villous tissue from IUGR placentas.[112]

The elevated trophoblast apoptosis observed in pregnancies complicated by preeclampsia and IUGR is thought to be the result of placental oxidative stress, which may, in part, be triggered by hypoxia.[114,115] Although there is some discrepancy as to whether trophoblast cells are sensitive to hypoxia-induced apoptosis,[59,116,117] hypoxia induces apoptosis in other cell types[118,119] and the mechanism by which hypoxia mediates its effects has been shown to involve the mitochondrial pathway.[120] Indeed, it was previously shown that hypoxia enhances apoptosis in term trophoblasts by decreasing the expression of Bcl-2 while increasing the expression of p53 and Bax and that EGF significantly lowered the level of hypoxia-induced apoptosis.[116] Moreover, a subsequent study with a first trimester extravillous trophoblast cell line demonstrated that caspase-3 and caspase-9 activation increases over time under hypoxic conditions and that EGF protects trophoblast cells from hypoxia by preventing the cleavage of caspase-3 even in the presence of a phosphatidyl-inositol-3-kinase (PI3K) inhibitor, suggesting that EGF exerts its anti-apoptotic effect independently of the PI3K/protein kinase B (Akt) survival pathway.[121]

The Future of Trophoblast Apoptosis

Regardless of whether apoptosis is triggered by death receptors, as a result of an intracellular signal such as DNA damage or in response to an exogenous stimulus, cells utilize common pathways, particularly in the execution phase, to undergo cell death. Although the incidence of apoptosis has been shown to be greater in villous trophoblast of third trimester placentas compared to that of first trimester placentas,[7,8] suggesting that placental apoptosis is developmentally regulated, relatively little is known about what controls apoptosis in the placenta. More mechanistic studies should enhance our understanding of how trophoblast cell apoptosis is regulated during normal pregnancy and may provide new therapeutic approaches for the treatment of pregnancy-related diseases associated with increased trophoblast apoptosis.

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CHAPTER 6

Macrophages and Pregnancy

Gil Mor, Roberto Romero and Vikki M. Abrahams

Abstract

During implantation, apoptosis is critical for the appropriate tissue remodeling of the maternal decidua and invasion of the developing embryo. Yet the regulation of apoptosis is also imperative for a successful pregnancy. The quick and effective removal of apoptotic cells by tissue macrophages represents an essential process, which prevents the release of self-antigens and, in the case of pregnancy, paternal alloantigens. Recent studies have shown that the process of apoptotic cell clearance is not a neutral event, but rather an active one which induces macrophage production of anti-inflammatory cytokines and survival factors. Therefore, apoptotic cell clearance is necessary for the resolution of inflammatory conditions, which during pregnancy could have lethal consequences. The function of the maternal immune system during implantation and throughout pregnancy is, thus, an important area of investigation. This review will discuss the role of decidual macrophages in apoptotic cell clearance during pregnancy.

Introduction

So far, the role of the maternal immune system during pregnancy has focused mainly on the aspect of immune tolerance towards the invading trophoblast and, therefore, the fetus. While this is a critical aspect of reproductive immunology, it is also important to consider the function of the maternal immune system in the promotion of implantation and maintenance of pregnancy. This review will focus on decidual macrophages and their role in the clearance of apoptotic cells during pregnancy.

Leukocytes at the Implantation Site

During normal pregnancy the decidua is populated by a variety of leukocytes.[1] However, cells of the innate immune system seem to dominate this tissue since the levels of lymphocytes are relatively low (1-3%).[2] At the time of implantation, many of the leukocytes are NK cells, expressing a phenotype distinct from those found in the periphery.[3] As gestation proceeds, NK cell numbers decline and at term these leukocytes are absent.[4] Macrophages constitute 20-30% of the decidual cells at the site of implantation[2,5-7] and unlike NK cells, remain high throughout pregnancy.[2,8] This evidence suggests that the innate immune system is not indifferent to the fetus and may play a role, not only in host protection to infections, but also as important factors in the fetal-maternal immune adjustment. A significant aspect in this process is the establishment of an adequate microenvironment that will promote cell growth and inhibit harmful inflammatory immune reactions.

Microenvironment of the Implantation Site

The local environment of the maternal-fetal interface is characterized not only by the cell types present, but also the soluble factors produced therein. The production and effects of

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cytokines at the implantation site is important for the regulation of trophoblast cell growth, differentiation and invasion.[9-11] In normal pregnancies, particularly at the maternal-fetal interface, anti-inflammatory Th-2 cytokines predominate.[12,13] Therefore, an appropriate balance between pro-inflammatory and anti-inflammatory cytokines is thought to be crucial for determining the success or failure of a pregnancy.[11] It is currently believed that for the continuous normal development of pregnancy, production of pro-inflammatory Th-1 cytokines such as interleukin-2 (IL-2), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) is suppressed, whereas production of anti-inflammatory cytokines such as, IL-4, IL-6 or IL-10 is enhanced.[12] Placental and decidual tissues from normal pregnancies have been shown to express both pro- and anti-inflammatory cytokines.[14-17] However, pro-inflammatory cytokines appear to be potentially harmful to pregnancy, since excess production of TNF-α or IFN-γ has been associated with preterm delivery.[17-20] Similarly, low levels of decidual IL-4 and IL-10 have been observed in women suffering from unexplained recurrent abortions and where spontaneous abortion has occurred during the first trimester of pregnancy.[19] Immune cells, particularly macrophages, are a main source of cytokines and growth factors and we postulate that these cells contribute to maintaining the appropriate balance of Th-1 and Th-2 cytokines at the placental bed. How this occurs may be a reaction of these immune cells to the natural turnover of the invading trophoblast population.

Apoptosis and Implantation

Apoptosis, or programmed cell death, is a natural mechanism by which the body eliminates unnecessary or potentially dangerous cells in order to maintain normal tissue function. During implantation, apoptosis is important for the appropriate tissue remodeling of the maternal decidua and invasion of the developing embryo.[7] Although, first trimester trophoblasts are resistant to Fas-stimulation, apoptosis has been described in the trophoblast layer of placentas from uncomplicated pregnancies throughout gestation. This suggests that there is a constant cell turnover at the site of implantation which is necessary for the appropriate growth and function of the placenta.[21-23] In addition, the incidence of trophoblast apoptosis is higher in third trimester villi compared with first trimester placenta,[24] suggesting that elevated placental apoptosis may be involved in the process of parturition. In pregnancies complicated by preeclampsia or intrauterine growth restriction (IUGR), there is a greater incidence of placental apoptosis in the first trimester, which is accompanied by insufficient trophoblast invasion.[23,25] In addition to apoptosis, several mechanisms have been described to limit extravillous trophoblast invasion into the uteroplacental arteries. These include reduced expression of integrin α1β1,[26] absent expression of vascular endothelial cadherin,[26] decreased secretion of matrix metalloproteinase 9 (MMP-9),[27] low cell surface plasminogen activator activity, and reduced expression of HLA-G.[28] The observed reduction of these factors, which are necessary for trophoblast differentiation and invasion, is compatible with the hypothesis of increased apoptosis in cases of IUGR, since once cells enter the apoptotic cascade, proteins are down regulated at the level of transcription.[29] This indicates that the regulation of placental apoptosis is essential for the normal physiology of pregnancy. However, cell death by apoptosis is not the end of the story. The clearance of apoptotic bodies represents a critical step in tissue homeostasis by preventing the release of intracellular contents which may initiate an inflammatory reaction and possibly cause tissue damage.

Clearance of Apoptotic Cells

Different morphological changes accompany the execution of the apoptotic program. Cells first become round and detach from their neighbors. Then, condensation of both the nucleus and cytoplasm occurs without major modification to the other intracellular organelles. Following condensation, nuclear fragmentation and membrane blebbing is observed, resulting in the formation of apoptotic bodies with intact membranes. These morphological changes are a translation of the biochemical modifications, mediated by the activation of the caspase cascade, that are occurring inside of the cell. Another important cellular change that occurs during

apoptosis is the redistribution of membranal proteins, which will allow macrophages to recognize apoptotic cells and direct the phagocytic process. Several receptors have been implicated in the recognition and engulfment of apoptotic cells,[30,31] suggesting that the process of phagocytosis is well regulated and functionally relevant.

As discussed above, implantation and trophoblast invasion is characterized by a progressive, continuous induction of apoptosis in the maternal tissue surrounding the fetus.[32] During this period, numerous macrophages are present at the implantation site and this was originally thought to represent an immune response against the invading trophoblast. However, we propose that this may not be the case. We suggest that macrophage engulfment of apoptotic cells prevents the release of potentially pro-inflammatory and pro-immunogenic intracellular contents that occurs during secondary necrosis (See Fig. 1). Due to the allogenic nature of the placenta, this process may be essential for the well-being of the fetus. Trophoblast cells are carriers of proteins, which are antigenically foreign to the maternal immune system and if released, as result of cell death, may initiate or accelerate immunological responses with lethal consequences for the fetus. Therefore, the appropriate removal of dying trophoblast cells prior to the release of these intracellular components is critical for the prevention of fetal rejection. Macrophages are a key cellular constituent of this process.

Macrophages and Clearance of Apoptotic Cells

The removal of cellular debris, generated as a result of apoptosis, is a challenging task that must be performed to maintain cellular homeostasis. This clearance process, far from being the end, represents an active and coordinate event which sends specific signals to the remaining cells, either for survival or death.[33] The removal of apoptotic bodies is neither a neutral nor a passive process, but rather an active physiological event that may influence not only immune responses, but also the proliferation and differentiation of surrounding cells.[31] Consequently, a signal from a macrophage to the wrong cell type may have profound consequences for the normal physiology of the tissue.[34]

Recent reports have shown that binding to, and ingestion of, apoptotic cells by phagocytes can result in active immunosuppressive and anti-inflammatory responses. Voll et al[35] found that monocytes secretion of TNFα was inhibited, while production of TGFβ and IL-10 was increased following coculture of monocytes with apoptotic lymphocytes. Furthermore, in vivo studies have clearly demonstrated that the TGFβ released by macrophages ingesting apoptotic cells, has anti-inflammatory effects in inflamed peritoneum and lungs.[36] Furthermore, the capacity for macrophages to influence cell death may be regulated by the extent of uptake of apoptotic cells.[31] Duffield et al demonstrated that the capacity of macrophages stimulated with IFNγ and LPS or TNFα to induce glomerular cells apoptosis could be suppressed by the uptake of apoptotic cells.[37]

Macrophages at the Maternal-Fetal Interface

Histological analysis of normal placental beds shows the presence of large number of macrophages which, in the majority of cases, are localized to the vicinity of apoptotic cells. Indeed, macrophages are one of the major cell types in both the maternal and fetal compartments of the uteroplacental unit.[38] In humans, during the first weeks of implantation, macrophages are found in high numbers in the maternal decidua and in tissues close in proximity to the placenta.[39] Similarly, in rodents macrophages accumulate at or near the implantation site.[40] The dense macrophage infiltration at the maternal fetal interface suggests that these cells not only to perform their usual immunological tasks but are also involved in specific pregnancy-associated functions.[41] Hunt and coworkers have implied that maternal macrophages assist in the tissue remodeling necessary to accommodate expansion of extraembryonic tissue.[42] However, macrophages are not merely scavengers of dying cells, but also actively orchestrate apoptosis of unwanted cells during tissue remodeling.[31] Macrophages synthesize and secrete cytokines and growth factors, which govern the local cellular and tissue interactions.[39,42-44] They also respond to hormonal factors affecting their function and survival.[45,46]

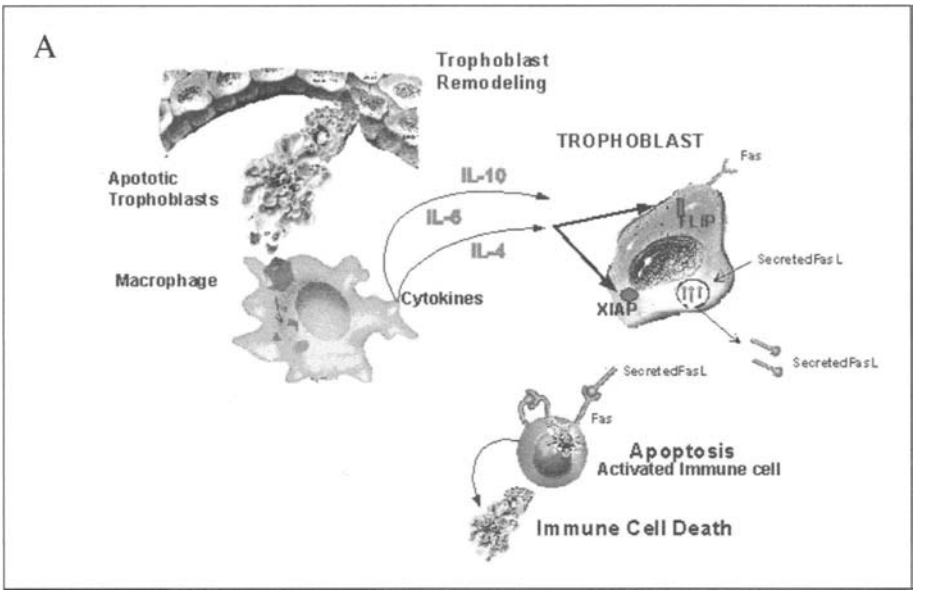

Figure 1A. Effect of clearance of apoptotic cells by macrophages. Clearance of apoptotic cells induces the expression, by macrophages, of anti-inflammatory cytokines with protective effects on trophoblast survival and immunological tolerance.

Numerous findings indicate that the capacity for macrophages to influence cell death is regulated by the extent of uptake of apoptotic cells.[31] For example, the cytolysis of tumor cells by activated macrophages is inhibited by the ingestion of apoptotic but not necrotic cells.[47]

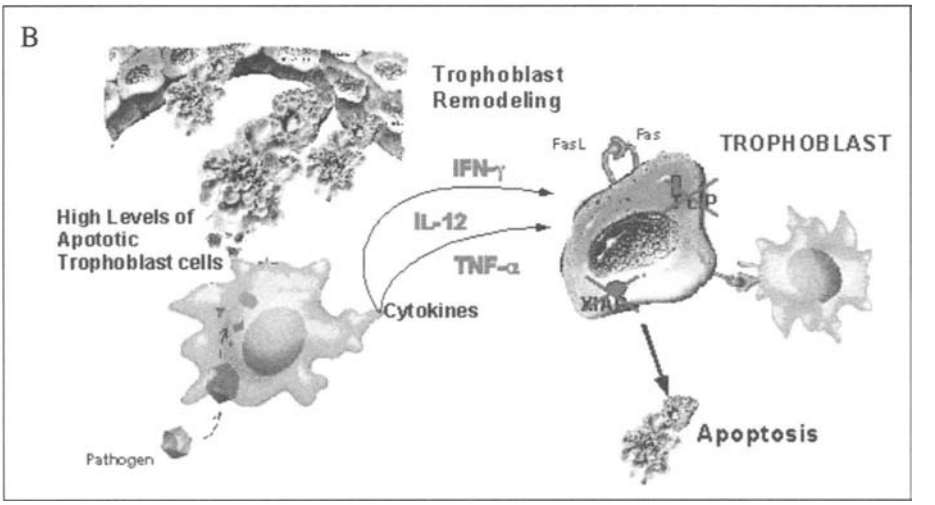

Figure 1B. Effect of clearance of apoptotic cells by macrophages. Changes in the cytokine milieu, owing to elevated levels of apoptotic bodies and inefficient clearance, will result in a pro-inflammatory microenvironment that in turn may result in changes in trophoblast resistance to Fas-mediated apoptosis and the maternal immune system

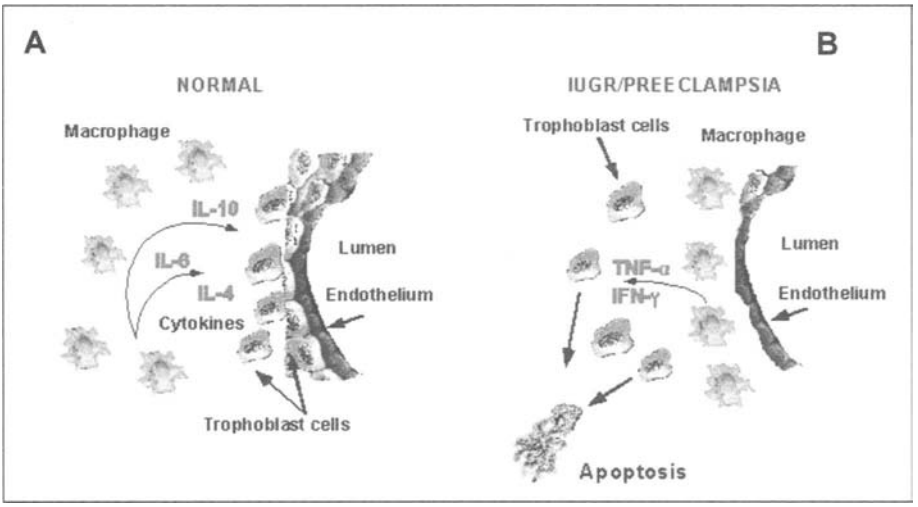

Figure 2. Differential distribution of macrophages in normal pregnancy and pregnancy complicated with preeclamsia and IUGR. While in normal pregnancies macrophages are located in the stroma surrounding the transformed spiral arteries and extravillous trophoblast (A), in preeclampsia macrophages are found within and around the spiral arteries separating the walls of the artery from the trophoblast cells (B). In the normal condition, macrophages promote trophoblast survival, while in the pathologic state induce apoptosis.

Similarly, during embryo implantation, uterine epithelial cells surrounding the blastocyst undergo apoptosis and may form an anti-inflammatory environment by increasing Th-2 type cytokines. This may explain the surprising cohabitation of macrophages and trophoblast cells at the implantation site. As stated earlier, the type of cytokines produced by a macrophage depends on its activation state.[48] We suggest that during normal pregnancy, the uptake of apoptotic cells suppresses macrophages from secreting pro-inflammatory cytokines such as TNF-α and IFN-γ, and promotes the release of Th-2 anti-inflammatory and immunosuppressive cytokines (Fig. 1A). In pregnancies complicated with preeclampsia or IUGR, we propose that activated macrophages secrete pro-inflammatory cytokines such as TNF-α and IFN-γ, which induce apoptosis in extravillous trophoblast (Fig. 1B). This hypothesis is supported by a recent report of Pijnenborg et al[49] who found a higher incidence of cell clusters secreting TNF-α in the placental bed of patients with severe forms of preeclampsia. These cells are likely to be macrophages.

Macrophages are also found near the spiral arteries during trophoblast invasion and transformation. Previous studies of placental bed specimens by ourselves and others, have demonstrated changes in the distribution of macrophages during pathological conditions such as preeclampsia.[50,51] While in normal pregnancies macrophages are located in the stroma surrounding the transformed spiral arteries and extravillous trophoblast (Fig. 2), in preeclamsia macrophages are found within and around the spiral arteries separating them from the trophoblast cells (Fig. 3). Their distribution resembles a barrier between the invading trophoblast and the spiral arteries (Figs. 3 and 4). Moreover, Resiter et al[51] have reported that macrophages residing in excess in the placental bed of preeclamptic women are able to limit extravillous trophoblast invasion of spiral arteries segments through apoptosis mediated by the secretion of TNFα. We propose a differential role for uterine macrophages during trophoblast invasion and differentiation, according to their activation status. In normal pregnancies, macrophages function as support cells by facilitating trophoblast invasion through the placental bed. In pathological conditions, macrophages function as a barrier for trophoblast invasion and differentiation by inducing trophoblast apoptosis and therefore preventing the transformation of spiral arteries (Fig. 4).

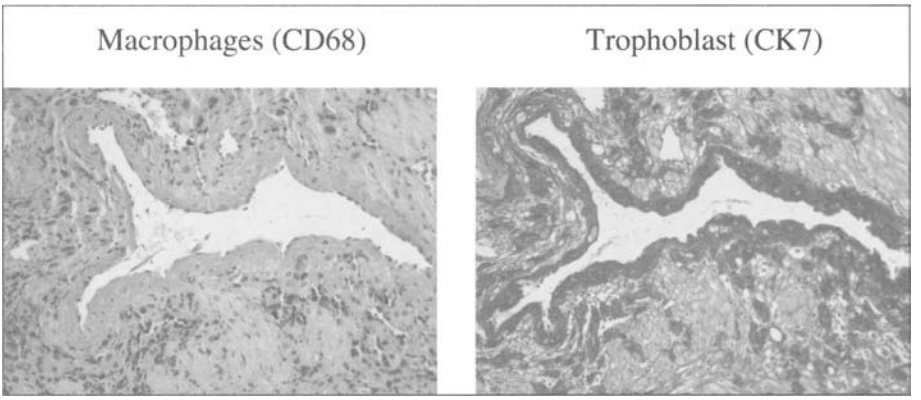

Figure 3. Localization of macrophages and trophoblast cells in normal pregnancy. Spiral artery in a placental bed biopsy specimen from normal pregnant women at 40 weeks of gestation (CD68 for macrophages and cytokeratin-7 for trophoblast). Trophoblast cells (brown color, right panel) are infiltrating the wall of the spiral artery. Macrophages (left panel) are localized in the stroma surrounding the trophoblast cells.

Role of Apoptotic Cell Phagocytosis in Pregnancy-Associated Diseases

The anti-inflammatory response following phagocytic clearance of apoptotic cells may be perturbed in disease processes. Anti-phospholipid antibodies in apoptotic cells may bind to surface expressed Fc receptors on macrophages resulting in secretion of pro-inflammatory cytokines such as, TNF-α.[52] Therefore, during pregnancy, cytokine production by macrophages and other cells at the maternal-fetal interface may be drastically altered.[53] The results of our studies concord with this concept and indicate that enhanced levels of pro-inflammatory macrophage products increase Fas expression and intensify trophoblast sensitivity to Fas-mediated apoptosis.[54,55] We have also demonstrated that the factors produced as a result of phagocytosis

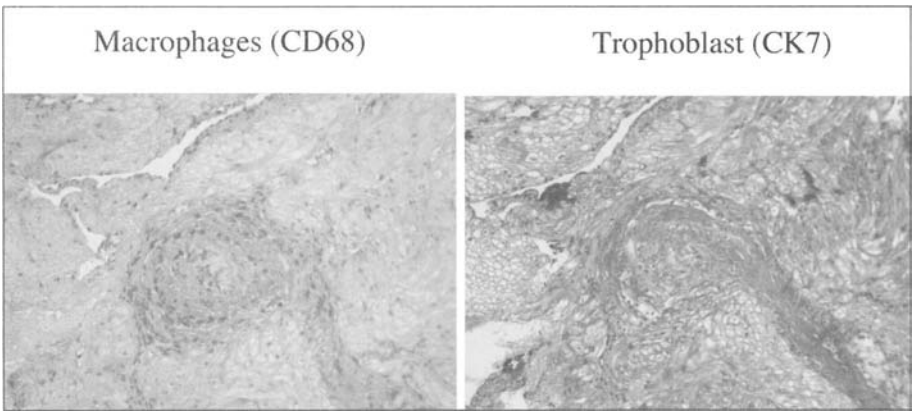

Figure 4. Localization of macrophages and trophoblast cells in preeclamptic pregnancies. Spiral artery in a placental bed biopsy specimen from a patient with preeclamsia (same gestational age as in (Figure 3)). (CD68 for macrophages and cytokeratin-7 for trophoblast). Trophoblast cells (brown color, right panel) have failed to induce transformation of the spiral arteries and are scattered distributed in the stroma, away from the spiral artery. Macrophages (left panel) are localized surrounding the spiral artery creating a barrier between the trophoblast and the artery.

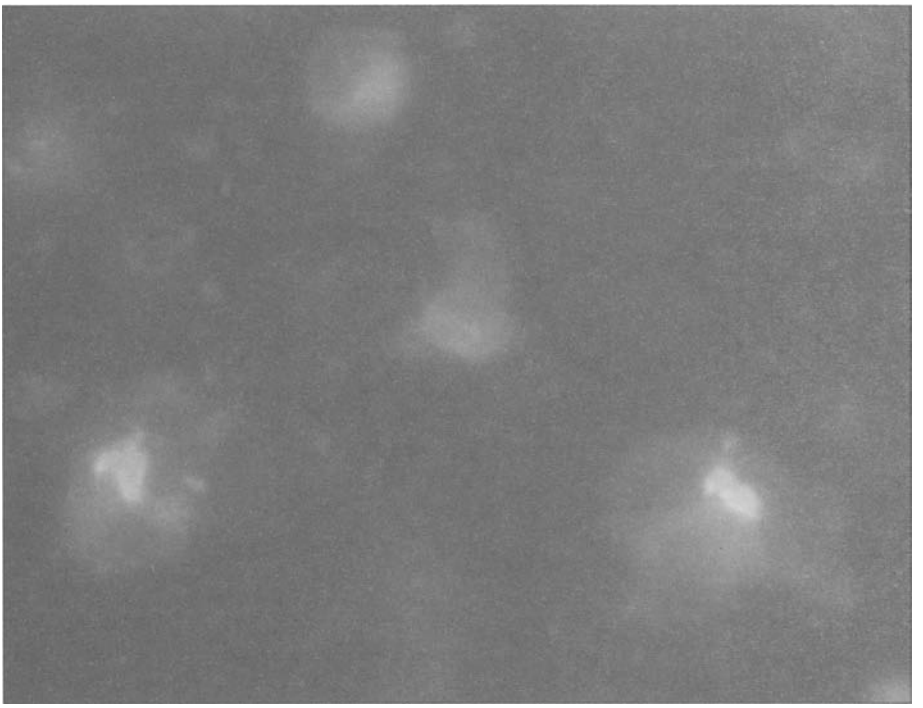

Figure 5. Macrophages phagocytose apoptotic first trimester trophoblasts. Apoptotic trophoblasts cells (green) are engulfed by THP-1-differentiated macrophages (red).

of apoptotic cells have a significant impact on viable trophoblast cells and is dependent on the levels of apoptotic cells. We have developed an in vitro system consisting of monocyte-derived macrophages and apoptotic first trimester trophoblast cells. We find that macrophages successfully engulf apoptotic trophoblast cells (Fig. 5). Furthermore, trophoblasts cells treated with conditioned media collected from monocyte-derived macrophages, cocultured with low numbers of apoptotic trophoblast expressed the active form of XIAP, an inhibitor of apoptosis, and showed no caspase-3 activation. This supports the hypothesis that clearance of apoptotic trophoblast cells by macrophages may protect the expanding trophoblast population from cell death during pregnancy. In contrast, trophoblast cells treated with conditioned media collected from macrophages cocultured with high levels of apoptotic trophoblast cells, expressed the inactive from of XIAP and the active forms of caspase-3 (Fig. 6). This suggests that an excess of apoptotic bodies may stimulate macrophages to produce pro-apoptotic factors. Alternatively, apoptotic cells undergoing secondary necrosis as a result of a failure of efficient clearance may directly influence trophoblast cell viability. Therefore, an increase in the levels of trophoblast apoptosis, possibly as a result of infection, may initiate an inflammatory event that will further promote trophoblast cell death preventing normal trophoblast invasion, spiral arteries transformation and fetal survival. This may be the case in pathologies such as preeclampsia and IUGR.

Conclusion

Apoptosis or cell death is not the final stage during tissue development. The quick and effective removal of apoptotic cells by tissue macrophages represents a vital process in order to prevent the "leak" of self-antigens and such clearance promotes the production of survival factors. One of the key requirements of apoptotic cell clearance is the resolution of

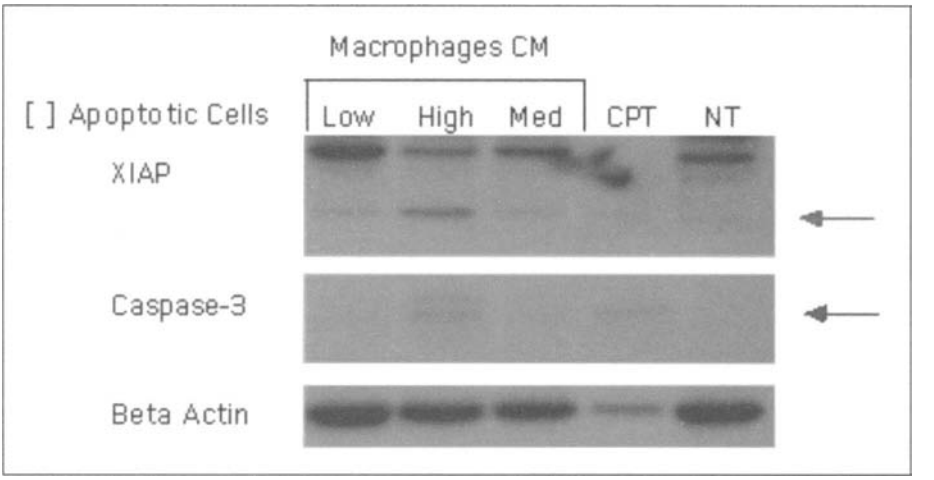

Figure 6. Phagocytosis of apoptotic cells by macrophages influences trophoblast apoptosis. Trophoblast cells were treated with macrophage condition media. Caspase 3 and XIAP expression was determined after a 24h incubation. NT: Control, CPT: Trophoblast cells treated with camptothecin. Low, High Med: Condition media from macrophages treated with either low, high and medium concentration of apoptotic cells.

inflammatory conditions, which, as in the case of pregnancy, may have lethal consequences. The clearance of the apoptotic trophoblasts at the implantation site by decidual macrophages and its effect on the well-being of the placenta throughout pregnancy is a novel area, warranting further investigation.

Acknowledgements

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CHAPTER 7

Potential Role of Glucocorticoids in the Pathophysiology of Intrauterine Growth Restriction (IUGR)

Seth Guller, Yuehong Ma and Men-Jean Lee

Abstract

Although the etiology of intrauterine growth restriction (IUGR) and preeclampsia (PE) remains unclear, most investigators attribute the initial "insult" to poor utero-placental perfusion due to defective trophoblast invasion that ultimately compromises fetal well-being.[1-3] The resultant hypoxia curtails the remodeling of uterine vessels by invasive cytotrophoblasts in the second trimester.[1,2] Our results suggest that mediators of fetal stress [i.e., glucocorticoids (GC)] may in fact alter placental gene expression and contribute to the destruction of the placental villous network in pregnancies with IUGR/PE. We will present a molecular model through which GC, induced in response to fetal stress, promotes the placental villous damage observed in pregnancies associated with IUGR/PE. This model incorporates the roles of trophoblast plasminogen activator inhibitor (PAI)-1, mesenchymal extracellular matrix (ECM) proteins, and their regulation by transforming growth factor (TGF)-β. We will employ the term "IUGR/PE" to describe those pregnancies with severely growth-restricted fetuses may also complicated by maternal PE. These conditions frequently coexist, and a review of the literature suggests that this placental pathology may be associated with both IUGR and PE. Furthermore, considerable attention has been given to the role of exogenously administered and stress-induced endogenous increases in fetal GC and the development of IUGR. There is mounting evidence that aberrant elevations in GC during fetal life and/or IUGR may result in fetal programming of chronic diseases of adulthood such as diabetes, coronary artery disease, and hypertension.

Excess Placental Fibrin and ECM Proteins Are Noted in Pregnancies with IUGR/PE

The most severe cases of IUGR/PE are associated with 40 to 50 percent fetal mortality and are usually characterized by absent or reversed end diastolic flow (AEDF or REDF) in the umbilical artery.[4] Placentas delivered from pregnancies with AEDF show a higher frequency of maldeveloped, elongated, poorly branched, and poorly vascularized terminal villi, the principal sites of nutrient and oxygen exchange between mother and fetus.[5,6] Although the precise etiology of these changes in placental structure is not known, it is generally thought to result from defective cytotrophoblast invasion in the first two trimesters of pregnancy.[1-3] Histological studies of IUGR/PE placentas have revealed two specific biochemical changes relative to controls matched for gestational age; excessive perivillous (i.e., in the intervillous space) deposition of fibrin and up-regulation of ECM proteins in the villous core.[7-9] Immunohistochemical

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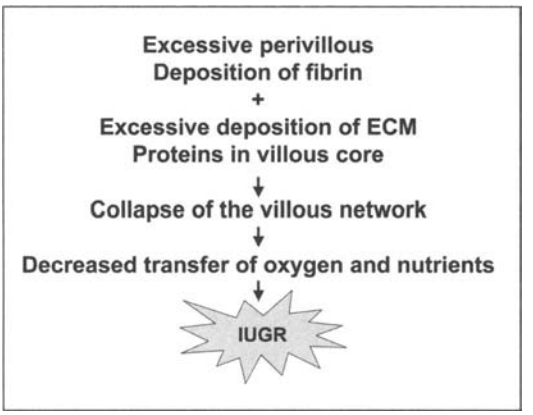

Figure 1. Placental pathophysiology in IUGR. Based on several studies, we and others suggest that aberrant deposition of intervillous fibrin and intravillous ECM proteins lead to collapse of the villous network (infarction) in pregnancies associated with severe IUGR. This results in a reduction in the transfer of oxygen and nutrients from mother to fetus.

analyses have demonstrated thickened basal lamina and increased expression of collagen III and IV and laminin in the core of the placental villus in pregnancies with IUGR/PE.[7] In this same study, electronmicroscopy identified placental mesenchymal cells (PMCs) as the likely source of enhanced ECM protein expression noted in IUGR/PE placentas.[7] Furthermore, in pregnancies with IUGR/PE, excessive perivillous deposition of fibrin and intravillous ECM proteins was associated with extensive placental villous damage (increased prevalence of white infarcts or collapsed villi, necrosis, and fibrosis) and impairment of nutrient transport.[7-10] Thus, it is likely that hyper-accumulation of perivillous fibrin and placental ECM proteins in pregnancies associated with IUGR/PE disrupts placental architecture, collapses the villous network, and irreversibly restricts the flow of nutrients between mother and fetus (Fig. 1). We do not propose that excessive deposition of fibrin and ECM proteins cause IUGR, but rather that they play a critical role in the pathophysiology of these pregnancies. In addition, we acknowledge that there are several other potential cellular mechanisms of placental damage in pregnancies with IUGR and PE including apoptosis, hypoxia and reperfusion injury,[11-14] all of which may be affected by GC. However, for the purpose of this report we are limiting our discussion to a potential unifying mechanism underlying excessive placental fibrin and ECM protein deposition in these pregnancies.

Plasminogen Activator Inhibitor (PAI-1): Role in Fibrin Deposition in Pregnancy

PAI-1 is a 52 kD protein that is a member of a serpin (serine protease inhibitor) family of protease inhibitors that also includes PAI-2 and PAI-3.[15-17] PAI-1 is the primary inhibitor of fibrinolysis based on its high affinity suppression (K_I ~1 nM) of tissue type plasminogen activator (tPA).[16,17] PAI-1 forms a 1:1 molecular complex with tPA and inhibits tPA-mediated conversion of plasminogen to plasmin, the major fibrinolytic factor.[15-17] PAI-1 was originally described as an endothelial cell protein,[18] but later reports revealed PAI-1 to be synthesized by many cell types, including those found at the uterine-placental interface, e.g., trophoblasts and decidual cells.[19-21] Interaction with the ECM protein vitronectin stabilizes PAI-1 and may localize it to areas of thrombosis.[22,23] Excessive production of PAI-1 would be expected to compromise fibrin clearance when the clotting cascade is activated. Pregnancy is considered to be a thrombophilic state based on the elevation of several plasma coagulation factors (e.g., factor VII, VIII, X and fibrinogen) in maternal sera across gestation.[24-27]

Several lines of evidence implicate placental PAI-1 in the excessive levels of perivillous fibrin deposition and placental damage noted in pregnancies with IUGR and PE.[28-34] Levels of PAI-1 in maternal sera from IUGR/PE pregnancies are elevated, and plasma levels of PAI-1 correlated with the severity of placental damage.[29,31-33] Elevated levels of PAI-1 mRNA (by Northern blotting) and protein (by immunohistochemistry) are observed in IUGR/PE placentas compared to gestational age-matched controls.[28,35] Syncytiotrophoblasts (SCTs), the placental cell type that line the intervillous space and are in direct contact with maternal blood, were shown by in situ hybridization to be the likely source of elevated placental PAI-1 in pregnancies associated with IUGR/PE.[28,34] In uncomplicated pregnancies, PAI-1 expression is limited to extravillous and invasive trophoblasts and not SCTs,[36,37] indicating that syncytial expression of PAI-1 is pathological. Further evidence for a role of PAI-1 in extravillous trophoblast function is the recent result showing that the presence of function blocking anti-PAI-1 antibodies promoted invasiveness of human first trimester extravillous trophoblasts.[38] Of note, infarcted areas of IUGR/PE placentas have been demonstrated to manifest the highest levels of immunoreactive PAI-1in SCTs.[28,34] In contrast, lower levels of PAI-2 were found in placentas and maternal sera in pregnancies with IUGR/PE compared to controls.[28,34,35] Compared to PAI-2, PAI-1 is 1000-fold more inhibitory of tPA-mediated fibrinolysis.[15-17] These observations indicate that PAI-1 is the dominant regulator of fibrinolysis in the intervillous space in pregnancies with IUGR/PE. These results suggest that the localization as well as the elevation of placental PAI-1 is important in the genesis of placental damage and thrombotic sequelae associated with IUGR/PE.

Role of TGF-β and Hypoxia on the Expression of PAI-1 and ECM Proteins

TGF-β, is a cytokine family of three closely related peptides (TGF-β1, β2, β3), that act through membrane receptors (types I and II).[39] Downstream signaling of TGF-β is mediated by phosphorylated Smads (Sma and MAD gene homologues in *C. elegans* and *D. melanogaster*) that bind to TGF-β-responsive 5'upstream sequences.[39] Smad binding sites have been identified in the promoters of genes including PAI-1.[39-41] The 3 TGF-βs[42-44] and types I and II receptors[45-47] are expressed by human placenta. Early studies showed that TGF-β treatment stimulated the synthesis of ECM proteins including fibronectin, collagens and their cell surface integrin receptors.[48-50] TGF-β was later found to be an important enhancer of PAI-1 expression in several cell types including HTR-8/SVneo cells,[51,52] a first trimester cytotrophoblast cell line. Autocrine regulation of first trimester cytotrophoblast proliferation and invasion by TGF-β has been proposed.[43]

Hypoxia up-regulates gene transcription through the binding of hypoxia inducible factor (HIF)-1 to hypoxia response elements (HRE) in the promoter region of several genes including erythropoietin,[53] heme oxygenase-1[54] and vascular endothelial cell growth factor.[55] HRE-like sequences have been identified in the PAI-1 promoter.[56] Graham and colleagues showed that both hypoxia and TGF-β up-regulate PAI-1 expression in HTR-8/SVneo cells.[57] Using an antibody that blocks the action of TGF-β_1 and β_2 they showed that the effects of hypoxia on PAI-1 expression were not mediated by TGF-β,[57] an important finding in light of previous studies showing that hypoxia up-regulates TGF-β expression in several cell types.[58-60] Hypoxia, like TGF-β, also enhances the expression of ECM proteins including collagen I, III and IV in lung parenchymal cells,[61] and collagen IV and fibronectin in mesangial cells,[62] and collagen I in cardiac fibroblasts.[63]

Evidence That Glucocorticoids Stimulate PAI-1 and ECM Protein Expression in Placenta by Enhancing the Action of TGF-β

As reviewed above, excessive production of placental PAI-1 has been associated with aberrant periplacental fibrin deposition in pregnancies complicated by preeclampsia PE and IUGR. We recently employed HTR-8/SVneo cells and primary cultures of term cytotrophoblasts as

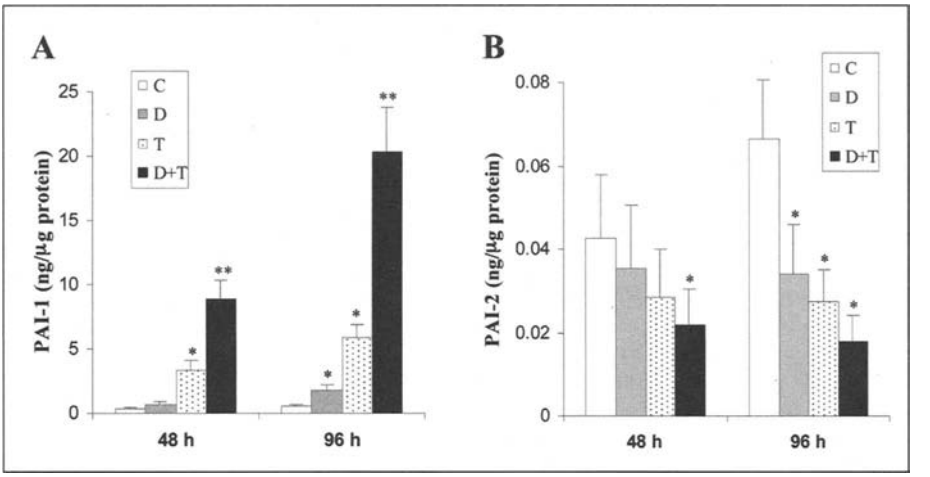

Figure 2. Regulation of PAI-1 and PAI-2 levels in HTR-8/SVneo cells by DEX and TGF-β. HTR-8/SVneo cells were maintained for 48 or 96 h in serum-free medium without (C) or with 100 nM DEX (D) and 1 ng/ml TGF-β (T) and levels of PAI-1 (Panel A) and PAI-2 (Panel B) in culture media were measured by ELISA in 5 independent experiments. Results are expressed as a mean ± SEM. *= P<0.05 vs control; **= P<0.05 vs the 3 other treatment groups (from ref. 64).

models for study of PAI-1 regulation by transforming growth factor (TGF)-β, and dexamethasone (DEX), a synthetic GC.[64] ELISA and assays revealed that DEX treatment significantly enhanced TGF-β effects on PAI-1 protein levels culture medium of HTR-8/SVneo cells and cytotrophoblasts several fold (Figs. 2 and 3). Conversely, DEX and TGF-β treatment suppressed PAI-2 levels in HTR-8/SVneo cells and did not affect PAI-2 levels in cytotrophoblasts. This indicated that the effects of DEX and TGF-β on PAI-1 expression in trophoblasts were specific. This result is interesting, because unlike PAI, PAI-2 is not suggested to be a critical

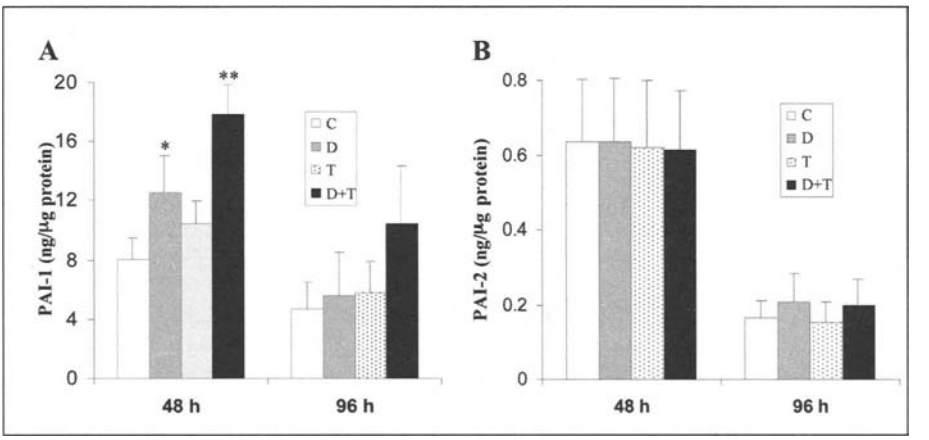

Figure 3. Regulation of PAI-1 expression in cytotrophoblasts by DEX and TGF-β. Primary cultures of cytotrophoblasts were maintained for 48 or 96 h in serum free medium without (C) or with 100 nM DEX (D) and 1 ng/ml TGF-β (T). Levels of PAI-1 (Panel A) and PAI-2 (Panel B) in culture media were measured by ELISA from cells isolated from 4 and 5 different placentas, respectively. Results are expressed as a mean ± SEM. *= P< 0.05 vs control; **= P< 0.05 vs the 3 other treatment groups (from ref. 64).

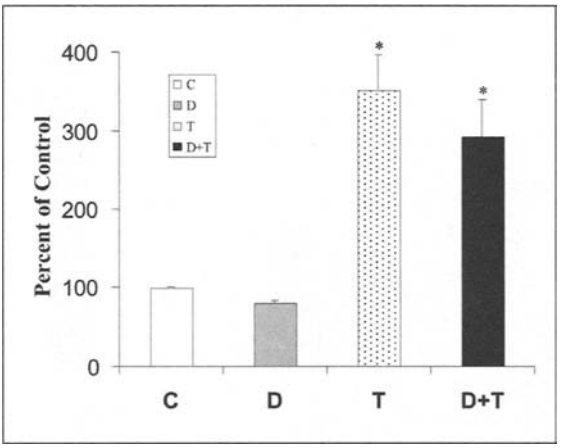

Figure 4. Regulation of PAI-1 promoter activity in HTR-8/SVneo cells by DEX and TGF-β. Transfected cells were maintained for 48 h in serum-free medium without (C) and with 100 nM DEX (D) and 1 ng/ml TGF-β (T). Levels of PAI-1 luciferase activity in cell extracts were determined and normalized to Renilla luciferase. Results are expressed as a mean ± SEM from 4 independent experiments. *= P< 0.05 vs control (from ref. 64).

regulator of fibrinolysis, but rather may perform an important anti-apoptotic role.[65,66] Northern blotting analyses have demonstrated that DEX stimulated levels of PAI-1 mRNA in HTR-8/SVneo cells and cytotrophoblasts several-fold.[64] Unexpectedly, PAI-1 promoter assays have revealed that TGF-β, but not DEX, enhanced PAI-1 expression in HTR-8/SVneo cells through a transcriptional mechanism, since DEX treatment suppressed PAI-1 promoter activity under both basal and TGF-β-stimulated conditions (Fig. 4). These results suggest that GC may alter fibrinolytic and invasive properties of trophoblasts through enhancing TGF-β effects on PAI-1 expression.

To elucidate potential mechanisms of ECM protein regulation in the stroma of the placental villus, we recently examined the interaction of GC and TGF-β in the modulation of ECM proteins in cultures of placental mesenchymal cells (PMCs, i.e., fibroblasts) isolated from human term placentas. Initial results obtained by ELISA showed that, similar to the effects observed for PAI-1 above, the combined treatment with dexamethasone (DEX) and TGF-β enhanced oncofetal fibronectin (FFN) protein levels in serum-free culture medium several fold (Fig. 5).[67] FFN was chosen as a model for study since it is a major ECM protein synthesized in vivo and in vitro by PMCs.[68,69] Real-time PCR analyses revealed a similar enhancement in levels of FN mRNA in cells treated with TGF-β and DEX (Fig. 6). Real-time PCR results have specifically demonstrated that DEX and TGF-β enhanced collagen (Col) I and Col IV expression, but did not affect levels of Col III or laminin, which is strongly suggestive of selective stimulation of ECM proteins. Similar results were obtained by Northern blotting.[67] In marked contrast to the results obtained with PMCs, we noted that DEX treatment suppressed FFN levels in untreated and TGF-β-treated cytotrophoblasts (Fig. 7), suggesting that GC and TGF-β modulate FFN expression in placenta in a cell type-specific manner. This indicates that GC and TGF-β may be key regulators of ECM protein synthesis in PMCs.

We predict that pregnancies associated with excessive placental exposure to GC and TGF-β would result in over-expression of ECM proteins by PMCs. Placentas from pregnancies with IUGR showed excessive villous fibrosis and ECM production by PMCs.[7-9] IUGR pregnancies typically have elevated umbilical cord Dopplers or absent diastolic flow which is indicative of increased placental vascular resistance.[70,71] The enhanced ECM protein production by PMCs that we have demonstrated in our study, and the resulting villous fibrosis seen on histological

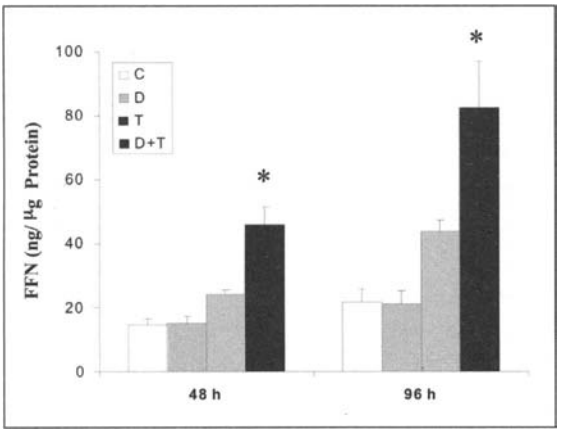

Figure 5. Effect of DEX and TGF-β treatments on oncofetal fibronectin (FFN) levels in placental mesenchymal cells (PMCs). PMCs were maintained for 48 or 96 h in serum-free medium without (designated "C" for control) and with 100 nM DEX (designated "D") and 1 ng/ml TGF-β (designated "T"). Levels of FFN in the culture medium were measured by ELISA and were normalized to cell protein. Results are presented as a mean ± SEM obtained in 4 independent experiments. *P < 0.05 vs all other groups by ANOVA (from ref. 67).

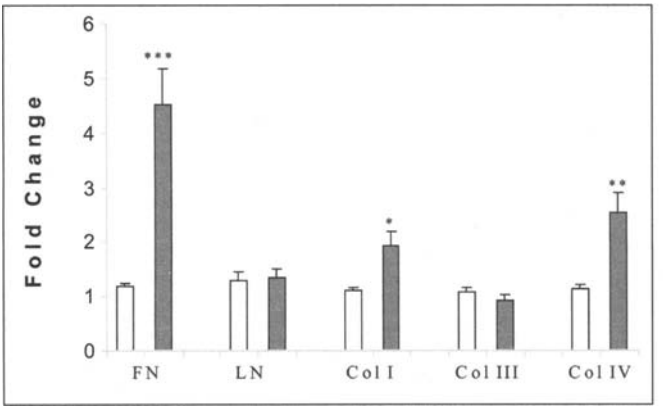

Figure 6. Analysis of DEX and TGF-β effects on ECM protein expression in PMCs by real-time PCR. Cells were maintained for 48 h in serum free-medium under two conditions: without (unfilled bars) or with 100 nM DEX and 1 ng/ml TGF-β (filled bars). RNA was extracted from cell lysates, converted to cDNA and levels of FN, laminin (LN), Col I, III, and IV expression were quantitated by real-time PCR analysis. Results are expressed as fold change relative to the calibrator (the lowest control value in each experiment), and are presented as a mean ± SEM obtained in 5 independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by Student's *t* test (from ref. 67).

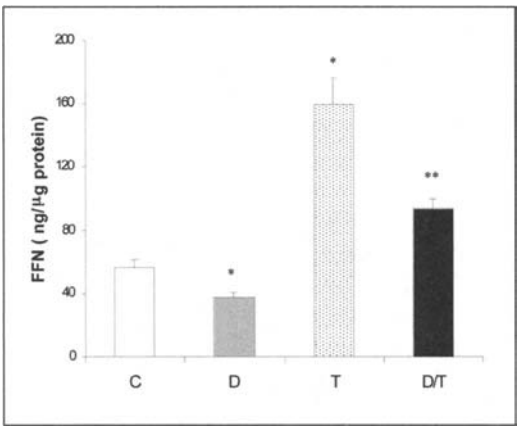

Figure 7. DEX and TGF-β effects on FFN levels in cultures of cytotrophoblasts (CTs). CTs were maintained for 48 h in serum-free medium in the absence (C) or presence of 100 nM DEX (D) and 1 ng/ml TGF-β(T) and the level of FFN in culture media was assessed by ELISA. Results are presented as a mean ± SEM from 6 culture wells obtained in a single experiment representing 3 identically conducted ones. *P < 0.01 vs control, **P < 0.01 vs control and TGF-β-treated group by ANOVA (from ref. 67).

examination,[7,9] may contribute to the clinical observation of increased placental vascular resistance. These changes in the villous stroma have been suggested to reduce the flow of nutrients from mother to fetus in pregnancies with IUGR.[7-9]

The level of GC in fetal sera has been found to be higher in pregnancies with IUGR compared to appropriately grown gestational age-matched controls.[72,73] The source of elevated periplacental GC in these pregnancies has been attributed to reduced placental levels of 11β-hydroxysteroid dehydrogenase-2 (the enzyme that irreversibly converts cortisol to the receptor inactive cortisone).[74,75] In addition, a direct correlation has been noted between the number of doses of antenatal GC given to women at risk for preterm delivery and the severity of villous fibrosis.[76]

Model of the Role of GC in Placental Damage in Pregnancies with IUGR/PE

Taken together, these studies suggest that the combined actions GC and TGF-β may play a critical role in the placental pathophysiology in pregnancies complicated by IUGR/PE. The model presented in Figure 8 proposes that reduced uterine-placental perfusion (hypoxia) initially activates stress pathways in the fetus leading to elevated levels of cortisol in fetal sera. Elevated periplacental levels of GC would then be expected to lead to excessive levels of fibrin and ECM proteins by specifically enhancing the effects of TGF-β on PAI-1 levels in trophoblasts and ECM proteins in PMCs, respectively. These changes would then promote villous collapse (infarction) and a further reduction in the flow of nutrients from mother to fetus.

Recent clinical trials have demonstrated that sustained exposure of the fetus and newborns to exogenous GC have been associate with IUGR.[77,78] Furthermore, prenatal exposure of the fetus and newborns to exogenous GC in animal models have demonstrated decreased somatic growth of the fetus,[79] reduced glomerular numbers,[80] and decreased islet cell numbers in the

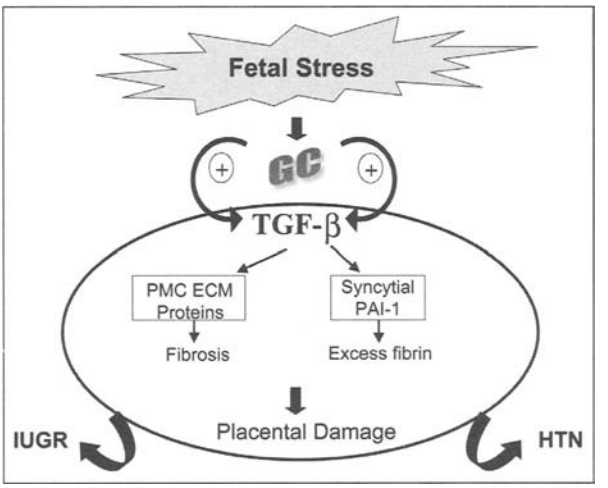

Figure 8. Model for the role of GC in placental pathophysiology in pregnancies complicated by IUGR/PE We suggest that elevated periplacental concentrations of GC due to fetal stress in pregnancies with IUGR/PE would promote excess fibrin and ECM protein expression in placenta through the enhancement of the actions of TGF-β. The resulting fibrosis and fibrin deposition is postulated to collapse the villous network resulting in severe placental damage. The reduction in the transfer of oxygen and nutrients from mother to fetus promotes IUGR. In addition, prenatal exposure of the fetus and newborn to IUGR and GC has been suggested to program the fetus for hypertension (HTN) and other chronic diseases as adults.

fetal pancreas,[81] all of which support the hypothesis of fetal programming of chronic diseases such as hypertension and diabetes. Therefore, GC appears to play a central role in the pathophysiology of IUGR and the programming of some major chronic adult diseases during fetal life.

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CHAPTER 8

NK Cells and Pregnancy

Mikael Eriksson, Satarupa Basu and Charles L. Sentman

Abstract

Natural killer cells are found in large numbers in the endometrium and decidua, and data suggest that NK cell functions and interactions with fetal-derived trophoblasts can have a profound impact on pregnancy. Altered NK cell numbers and activity have been associated with a variety of clinical conditions such as endometriosis, recurrent pregnancy loss, and preeclampsia. Uterine NK cells have a unique phenotype compared to blood NK cells and this is likely due to the specific tissue environment in which they reside. Specific chemokines produced by human endometrium and trophoblasts have been identified that may be responsible for recruitment of NK cells. Uterine NK cells can produce cytokines and may be an important part of vascular remodelling during placental development. This chapter summarizes current knowledge of NK cells in the uterus and their role in pregnancy and reproductive disorders.

Introduction

A mother's immunological tolerance to a semi-allogeneic fetus has long been puzzling for immunologists. The endometrium must be ready to respond to potential pathogen challenges, yet be able to control immune cell responses to allow the development of a semi-allogeneic fetus. Data have accumulated that there are extensive interactions between fetal cells and the mother's immune system. The immune system is often described as engaged in a struggle against all kinds of invaders, including allogeneic fetal tissue. However, an alternative viewpoint gaining support is that the maternal immune system may work together with fetal derived cells to create a hospitable environment in which the fetus may develop.

Natural killer (NK) cells account for a substantial presence in the uterus and data suggest that NK cell functions and interactions with fetal-derived trophoblasts can have a profound impact on pregnancy.[1] This review summarizes current knowledge of NK cells in the uterus and their role in pregnancy and reproductive disorders. We have focused on our understanding of human uterine NK (uNK) cells and also discuss particularly noteworthy findings from animal studies. Valuable data have been derived from animal studies, but significant differences exist in the structure of the placenta between species. Human NK cells are present in nonpregnant endometrium and are found throughout the endometrium and decidua, while NK cells are absent in mice prior to implantation and are restricted to the mesometrial triangle and decidua basalis. In humans, decidualization occurs during every menstrual cycle and there is extensive invasion of trophoblasts into maternal tissues. Decidualization occurs in mice after implantation, and trophoblast invasion into maternal tissue is minimal. These differences make extrapolation of data between species very difficult, so conclusions made in one species will not always be applicable to another.[2]

NK cells, also referred to as large granular lymphocytes or LGLs, have the ability to spontaneously kill tumor cells and secrete cytokines that can have powerful effects on immune- and

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nonimmune cells. As part of the innate immune system, NK cells are believed to act as sentinel cells prepared to attack foreign pathogens and promote host immune defenses.[3-5] Altered NK cell numbers and activity have been associated with a variety of clinical conditions involving reproductive organs and reproductive failure. Reduced NK cell activity is associated with an increase in the incidence of ovarian cancer, uterine cancer, and endometriosis.[6-9] Higher NK cell activity has been associated with recurrent pregnancy loss,[10,11] while NK cell function and specific NK cell receptors may be involved in the development of preeclampsia.[12,13] Thus, proper NK cell function in the uterus is an integral part of successful pregnancy.

Uterine NK Cells

Human NK cells are found in the blood, lymphoid organs, liver, and various mucosal tissues including lung, intestine, and uterus. Human peripheral blood NK cells can be divided into two major subsets based on the density of CD56 expression. $CD56^{dim}$ cells account for 90% of blood NK cells and have a high spontaneous lytic activity against tumor cells. This NK cell subset expresses CD16 (FcRγIII), killer cell immunoglobulin-like receptors (KIRs) and many of these cells express CD57.[14] The $CD56^{bright}$ NK cells account for approximately 10% of blood NK cells and these cells have less spontaneous lytic activity and have a high capacity to produce proinflammatory cytokines upon stimulation with monokines.[15] This NK cell subset expresses CD94/NKG2A/C dimers but contains only a small percentage of cells that express CD16 or KIRs and these cells lack CD57 expression.[16] Although comprising only a fraction of NK cells found in the peripheral blood, the $CD56^{bright}$ subset is the primary NK cell subset found in lymph nodes.[17,18] In addition to the markers mentioned above, there are a large number of other cell surface molecules that can be used to further subdivide NK cell subsets.[16,19]

Uterine NK cells have a unique phenotype compared to blood NK cells. uNK cells have previously been described as endometrial granulocytes, granular endometrial stromal cells, K cells, or even decidual NK (dNK) cells.[20] NK cells in the uterus express CD56 and CD94, few express CD16, and none express CD57 or CD8.[21,22] Most $CD57^{+}$ cells in the endometrium are T cells.[23] A significant percentage of uNK cells express KIRs on their cell surface.[22,24] Unlike blood NK cells, uNK cells express CD9 and CD69 on their cell surface.[22,25] Thus uNK cells have a cell surface phenotype that is unique. A summary of molecules and receptors on uNK- and blood NK cells is shown in Table 1. Recent molecular analysis of NK cells from decidua using gene array technology revealed 278 genes that were differentially expressed between decidual NK cells and blood NK cells.[25] This study indicated that decidual NK cells were more similar in their gene expression profile to $CD56^{bright}$ NK cells than to $CD56^{dim}$ NK cells. It remains unclear whether decidual NK cells are derived from blood NK cells that have differentiated in the unique environment of the decidua, or if they represent a special lineage of NK cells that are selectively produced for the decidua. Most evidence seem to favor the idea that the NK cells in the decidua are derived from uNK cells in the endometrium at the time of implantation or from newly recruited blood NK cells. These NK cells may respond to external signals to change their gene expression profile and presumably their function to respond to the unique requirements of the decidua. NK cells do not express receptors for estradiol and progesterone, so the action of sex hormones on NK cell function or recruitment is likely mediated via hormone action on other cells, such as fibroblasts or epithelial cells.[26,27] One report indicated that human uNK cells express glucocorticoid- and estrogen-β1 receptors but it is not known if this estrogen receptor functions in uNK cells.[26] NK cells isolated from the nonpregnant endometrium and decidua may not represent distinct phenotypes, but there may be a continuous process of differentiation such that the phenotype and functional activity of uNK cells may change as the local environment adapts to alterations in hormone levels and changes in the stromal cell environment.

One complication is that some studies have used NK cells isolated from first term decidua while others study NK cells from nonpregnant endometrium, and the hormonal status and local tissue microenvironment can be quite different between endometrium and decidua.

Table 1. Comparison between uterine NK (uNK) cells, CD56^{bright} and CD56^{dim} blood NK cells

Markers	uNK Cells[a]	Blood CD56^{bright}	Blood CD56^{dim}
CD56	++[b]	++	+
CD16	+/-	+/-	+
CD69,CD9,CD151	+	-	-
L-selectin	-	++	+/-
CD57	-	-	+
CD49a	++	-	-
CD44	++	++	+
PEN5-PSGL1	+/-	-	+
CD94	++	++	+/-
KIR[c]	+	+/-	+
KIR/NKAT 2	++	-	++
NKG2E/C/A	++	+	+/-
CXCR1	-	-	++
CXCR2	-	-	+
CXCR3	++	++	+
CXCR4	+	++	++
CXCR5	-	-	-
CCR1,CCR2,CCR3,CCR4	-	-	-
CCR5,CCR7	+	++	-
CX3CR1		+/-	++

[a] Some studies used NK cells from endometrium and others used decidual NK cells; [b]++: high expression; +: low expression; +/-: expression on subsets; -: no expression; [c]KIR: Killer cell immunoglobulin-like receptor family. References 15, 25 and 28-33.

Endometrial uNK cells are isolated from hysterectomy tissue or from microcurettage scraping of the endometrial lining. Even for endometrium from nonpregnant women, NK cells found in the proliferative phase and those from the secretory phase are in distinct tissue microenvironments and hormonal conditions. Thus, when reading the literature care must be taken to understand the tissue environment from where uNK cells have been isolated.

Recruitment of NK Cells into the Endometrium and Decidua

The human uterine endometrium is a complex mucosal tissue that is regulated by sex hormones throughout the menstrual cycle.[34,35] NK cell numbers are low early in the proliferative phase and increase as the menstrual cycle progresses.[36-38] NK cells may account for up to 70% of the leukocytes in the endometrium late in the secretory phase. At least two possible mechanisms have been hypothesized to be responsible for the drastic increase of uNK cells within the uterus: either in situ proliferation and/or a selective recruitment from the peripheral NK cell pool have been suggested.[34,39,40] Although proliferation could explain some of the increased numbers of NK cells, an active recruitment of these cells to the uterus is likely to play a major role. Data from murine studies indicate that uNK cells are derived from blood cells or bone marrow cells and not from NK cells within the uterus,[41] although in humans it has not been ruled out that NK cells may be renewed from a precursor within the noncycling part of the endometrium.

Given the increase in NK cells during the menstrual cycle and the role of sex hormones in modifying changes in endometrium, it has been proposed that sex hormones may regulate NK cell recruitment and expansion in the endometrium. Migration of cells into tissue involves a

variety of molecules, including adhesion molecules and chemokines. Cells in blood must bind to the endothelium and firmly adhere so that they can follow chemotactic gradients into tissues. Many blood cells express several different chemokine receptors and more than one may be involved in proper migration of cells into tissues. NK cells are found widely within nonpregnant endometrium and they can be associated with other leukocytes in small aggregates.[36,42,43] In the first trimester of pregnancy, uNK cells accumulate as a dense infiltrate around the trophoblast cells and spiral arteries.[20] As pregnancy progresses, uNK cells disappear from the decidua and are absent at term. These findings suggest that specific signals are involved in the recruitment and localization of NK cells within the uterus.

Blood NK cells express a variety of chemokine receptors, such as CCR5, CXCR3, and CXCR4, and specific migration of NK cells has been induced by chemokines in vitro.[31,38,44] uNK cells have been shown to express CXCR3, CCR5, and CCR7, but not other chemokine receptors.[29] Not all chemokine receptors have been analyzed on uNK cells and low levels of receptor expression may be functionally relevant. Kitaya and colleagues have examined the expression of chemokines within the endometrium at different stages of the menstrual cycle. They have reported that the expression of CCL4, CXCL9, and CXCL10 increases during the menstrual cycle and correlates with the increase in the number of NK cells in the endometrium.[45,46] The authors analyzed the expression of these chemokines by immunohistochemistry and found chemokines expressed in epithelial and perivascular stromal cells. It has been demonstrated that estrogen and progesterone are able to induce the expression of CXCL10 and CXCL11, but not other chemokines in primary human endometrium.[29] Several potential chemokine receptor-ligand pairs have been described in the decidua that could be involved in the leukocyte trafficking. Trophoblasts have been reported to express CXCL12, a ligand for CXCR4.[30] This study also shows that in vitro, CXCL12 preferentially recruits $CD56^{bright}$ NK cells compared to $CD56^{dim}$ NK cells. Placenta and endometrium have been shown to produce CCL3 that can recruit blood NK cells.[47,48] It has also been reported that $CD16^{-}/CD56^{bright}$ NK cells express chemokine receptor CCR5 and that its ligand CCL4 acts as a strong chemoattractant for these cells.[49] However, murine studies have shown normal uNK cell recruitment in mice deficient in CCR2 or CCR5.[50] Thus, a large number of studies show that the endometrium and decidua can produce chemokines that can recruit NK cells. Which chemokines are involved in NK cell recruitment in vivo remains to be determined, and it is possible that more than one chemokine is involved.[51] For example, CXCL10 induced by sex hormones in endometrium prior to implantation may recruit NK cells into the endometrium. As trophoblasts invade the decidua, chemokines derived from trophoblasts (e.g., CXCL12) may recruit uNK cells to reorganize placental arteries and facilitate trophoblast invasion of maternal tissue.

Studies have identified specific adhesion molecules involved in NK cell attachment to uterine endometrium. It has been established that L-selectins and $\alpha 4$ integrins are important for the binding of lymphocytes possibly facilitating extravasation into tissues.[52-55] Adhesion of $CD56^{bright}$ cells to uteri from pregnant or hormone-treated ovariectomized mice was enhanced through L-selectin- and $\alpha 4$ integrin-dependent mechanisms.[56] The ability of human $CD56^{bright}$ NK cells to adhere to mouse uteri is associated with successful outcome of in vitro fertilization (IVF) treatment.[57] The binding of NK cells also involved VCAM-1 and peripheral lymph node addressin. VCAM-1 is expressed at the site of trophoblast invasion and might allow NK cells to migrate continuously to these sites.[56,58,59] The proper migration of NK cells into the endometrium and localization to specific areas may be critical for NK cell function in the uterus.

Function and Regulation of uNK Cells

Human decidua contains a large number of NK cells and many of them localize near trophoblasts. Uterine NK cells have been proposed to have several different functions in pregnancy, including to: (i) help shield trophoblasts bearing paternal antigens from the maternal immune system, (ii) protect the mother from trophoblast invasion and limit expansion of trophoblasts, (iii) be involved in regulation and restructuring of maternal spiral arteries,

and (iv) be part of the innate defense system and protect against infection in the uterus. These ideas are not mutually exclusive and there is evidence to support each of them.

Regulation of uNK Cells

It has been proposed several years ago that successful pregnancy was associated with Th2-type environment rather than a Th1-type environment.[60] It has become clear that the reality is more complex, and there is evidence that both Th1 and Th2 cytokines are produced in decidua. Like blood NK cells, uNK cells can produce IFN-γ, GM-CSF, IL-10, TGF-β1, and IL-8 among other cytokines.[22,61-63] One study indicated that fresh decidual NK cells produce cytokines spontaneously.[64] uNK cell derived cytokines may have significant effects on decidualization and trophoblast invasion. Human endometrium expresses a wide range of angiogenic growth factors, and uNK cells may play an important role in abnormal endometrial angiogenesis.[65] uNK cells produce cytokines that are not normally made by blood NK cells such as angiogenic growth factors and leukemia inhibitory factor (LIF).[61,65] LIF is considered an essential cytokine for implantation in mice.[66]

Many different cytokines that alter NK cell function have been shown to be present in the human endometrium.[67,68] Endometrium is a source of both IL-15 and prolactin,[69-71] and both of these have been implicated in the proliferation and differentiation of uNK cells. There are data that uNK cells express prolactin receptors.[72,73] Evidence suggest that IL-15 is required by uNK cells for survival and proliferation.[74-78] In humans, IL-15 is present throughout the menstrual cycle, is increased during the secretory stage and early pregnancy, and is produced by stromal cells during decidualization.[69,79-81] It has been suggested that IL-15 expression in endothelium may be important for NK cell attachment, and perhaps IL-15 expression by decidual endothelium may be involved in specific localization of uNK cells close to spiral arteries.[59,82,83]

Several reports have observed effects of TGF-β on NK cells.[84-88] Members of the TGF-β family are powerful immunoregulatory molecules that act on a range of different immune cells and can demonstrate both activating and inhibitory function.[89-92] TGF-β proteins are produced as inactive precursors that bind to extracellular matrix and cell surface proteins where they can be activated.[93] The mechanisms that regulate and activate TGF-β proteins remain unclear. A recent paper shows that endogenous TGF-β-mediated inhibition is a mechanism that regulates uNK cell-derived cytokine production.[22] Another study reported that $CD56^+$ lymphoid cells in human first trimester pregnancy decidua are a source of novel transforming growth factor-β2-related immunosuppressive factors.[94] Furthermore, data suggest that TGF-β1 may be necessary to maintain pregnancy but may also be a risk factor for recurrent miscarriages.[95]

Molecules that differentiate NK cells in the endometrium are poorly understood. IL-11 has been implicated in the differentiation of uNK cells. IL-11 deficient mice have been shown to lack NK cells at implantation sites.[96] Although NK cell precursors could home to the uterus of IL-11 receptor α-/- mice, the NK cell defect was not thought to be due to the NK cells themselves but to signals within the environment. This study showed that the requirement for IL-11 to induce NK cell differentiation was tissue specific and that IL-11 signaling indirectly affected uNK cell differentiation. Many other factors are likely involved in uNK cell differentiation and they remain to be discovered.

NK Cell Trophoblast Interactions

NK cells and macrophages are found near trophoblasts in the first trimester of pregnancy.[20,97] Due to trophoblast expression of paternal antigens, low MHC expression, and their association with NK cells, it has been theorized that the trophoblast-NK cell interaction is a key part in the control of placental development. Trophoblasts express three different HLA molecules that

can interact with NK cell receptors, HLA-C, HLA-E, and HLA-G. CD158 receptors (members of the KIR family) bind to different HLA-C alleles and data suggest that this receptor-ligand interaction is involved in development of preeclampsia (see below). CD94/NKG2A dimers recognize HLA-E, and KIR2DL4 is expressed on uNK cells and recognizes HLA-G molecules. Many of these receptor-ligand interactions have been shown to alter NK cell function.

Much effort has focused on the susceptibility of trophoblasts to NK cell mediated cytotoxicity. Most studies have indicated that human trophoblasts are resistant to NK cell mediated killing.[98,99] Since most decidual NK cells express CD94/NKG2A dimers, it has been suggested that uNK cells could recognize HLA-E expressed on trophoblasts and inhibit NK cell activation. However, lysis of trophoblasts could not be induced even after blocking CD94/NKG2 receptors on NK cells or MHC class I molecules on trophoblasts.[98] Removing MHC class I on trophoblasts by acid treatment could not induce lysis by decidual NK cells. Lysis of trophoblasts could only be observed using IL-2 activated decidual NK cells. One study with murine trophoblasts demonstrated trophoblast rejection in nonpregnant allogeneic mice at extra-uterine sites, supporting the idea that trophoblasts are not inherently resistant to NK cells but that local factors in decidua protect them from immune attack.[100] The inability of trophoblast cells to act as target cells for NK cell cytotoxicity, even when known inhibitory receptors were blocked, implies that alternative inhibitory pathways exist or that trophoblasts are unable to trigger uNK cell activating receptors.

HLA-G is only expressed by trophoblasts and can interact with KIR2DL4 receptors on uNK cells. Data have suggested that HLA-G may inhibit NK cell activation, but ligation of KIR2DL4 led to stimulation of IFN-γ but not the triggering of cytotoxicity.[101] A recent publication suggested that membrane-bound HLA-G is able to stimulate purified uNK cells while it suppressed unfractionated mononuclear cell effector functions.[102] Because IFN-γ production by uNK cells is believed to be important for vascular remodelling (see below), the recognition of HLA-G on trophoblasts by uNK cells may be important for placental development. The fact that trophoblast protection from NK cell lysis appears to be largely independent of HLA class I expression suggests a modulatory function for HLA-E and HLA-G rather than a strict inhibitory effect on uNK cell effector functions. These receptor-ligand interactions may modulate uNK cell production of cytokines and angiogenic factors promoting trophoblast invasion and differentiation or tissue remodelling.[65]

NK Cell Regulation of Spiral Arteries

Key observations in murine studies have been made using mice deficient in NK cells or specific effector molecules. NK cell deficient mice do have offspring, but the litters are smaller in number. Although it remains unclear how much can be extrapolated to human placenta formation, careful analysis demonstrated ultra-structural consequences for implantation and placental formation in the absence of uNK cells in NK cell deficient mice.[41,103] Failure to sustain decidual integrity and loss of spiral artery modifications were the two key features affected in these mice. Additional studies also suggested that uNK cell derived IFN-γ was necessary for the proper structure of spiral arteries and placental formation.[104] In humans, uterine NK cells are nearly absent by 20 weeks of gestation, a time when vascular changes are complete. In the placenta, uNK cells are often seen in close proximity to transformed and nontransformed spiral arteries. Moreover, uNK cells can be a source of angiogenic growth factors such as NO synthase or Ang2 that may be involved in destabilizing blood vessel structure and promoting blood vessel remodelling.[65,105] The role of uNK cells in remodelling human spiral arteries remains unknown, but data suggest involvement of NK cell receptors in the development of preeclampsia that supports a role for human uNK cells in vessel remodelling (see next section).

NK Cells in Reproductive Disorders

Recurrent Pregnancy Loss

Recurrent pregnancy loss (RPL) is defined as three or more consecutive spontaneous abortions with the same partner. About half of all cases are explained by universally accepted aetiologies. Thus, proposed immune based aetiologies are being studied as a possible explanation for some of the other cases. Activated $CD56^+$ NK cells are present in increased numbers in peripheral blood in women with RPL.[106-110] NK cells produce IFN-γ which is a Th1 cytokine that is associated with RPL,[111,112] and LIF is down-regulated by IFN-γ.[113] It has been demonstrated that $CD56^{bright}$ NK cells are decreased in the secretory phase endometrium in patients with unexplained recurrent miscarriage[114] and in vitro fertilization-embryo transfer failure.[115] One possibility is that some of these patients have an influx of $CD56^{dim}$ NK cells that respond against fetal cells. There is evidence that NK cells and PBMCs from women with unexplained RPL respond to trophoblast extracts in vitro by proliferating and releasing embryotoxic factors that adversely affect embryo growth, while cells from women with normal pregnancies do not show such a response.[116] An increase in the absolute count of activated NK cells ($CD56^{dim}$/$CD16^+$/$CD69^+$) in the peripheral blood is associated with a reduced rate of embryo implantation during IVF treatment. Furthermore, women with high numbers of $CD56^{dim}$/$CD69^+$ blood NK cells who were able to achieve pregnancy had a significantly higher rate of miscarriage.[10] However, one must remember that the local maternal immune response to the fetus-derived cells may not be similar to the systemic response against fetal cells. Correlative data implicate NK cells in RPL, but a direct link has not been established. It is unclear whether an increase in NK cell cytotoxic activity and/or a decrease in other NK cell functions may account for some cases of RPL.

Endometriosis

Endometriosis is a condition where there is an ectopic occurrence of endometrial tissue, often in the peritoneal cavity. One theory is that endometrial cells move into the peritoneal cavity due to aberrant menstrual flow, and women with endometriosis frequently have difficulty becoming pregnant. Decreased NK cell activity in peripheral blood and peritoneal fluid of women with endometriosis has been established.[117] One mechanism for decreased NK cell activity may be increased KIR expression on NK cells, because increased expression of KIRs is generally associated with decreased NK cell activity.[8,9,118] Thus, NK cells may help prevent the development of endometriosis by removing endometrial cells in the peritoneum.

Preeclampsia

Preeclampsia is a serious complication of pregnancy and leads to high maternal blood pressure, elevated concentrations of urinary protein and poor fetal growth.[119,120] It can be caused by inadequate remodelling of spiral arteries and lead to the life-threatening condition of eclampsia. Alterations of NK cell function may be important in failure to reorganize blood vessels at the maternal-fetal interface and account in part for preeclampsia.[13] There is an increased incidence of preeclampsia in mothers who lacked most or all activating KIR genes (the AA KIR genotype) when the fetus possessed HLA-C genes of the C2 subgroup.[12] This combination leads to the greatest inhibition of NK cells via KIR-HLA-C interactions. These data support the idea that without the presence of activating receptors on NK cells, these cells may be prevented from completing the task of vasculature remodelling and result in preeclampsia.

Other studies suggest that preeclampsia is associated with a Th1/Th2 imbalance with a predominant Th1 immunity.[121] This may be considered a failure of tolerance (i.e., a decrease in NK cell inhibition) or an increase in NK cell activity. Serum levels of granulysin, a cytotoxic granule protein of natural killer (NK) cells and cytotoxic T lymphocytes, were significantly elevated in preeclampsia patients when compared to those in normal pregnancy subjects.[13] Preeclampsia was associated with an increased number of $CD56^{dim}$ NK cells in umbilical cord blood compared to the control group.[122] Thus, aberrant NK cell activity may have a significant role in the pathogenesis of this disorder.

Summary

Natural killer cells are found in large numbers in the endometrium and decidua and data suggest that NK cell functions and interactions with fetal derived trophoblasts can have a profound impact on pregnancy. Uterine NK cells have a unique phenotype compared to blood NK cells and this is likely due to the specific tissue environment in which they reside. We are beginning to understand the role of uNK cells, their cytokines and key receptors that allow them to function to establish a functional and supportive maternal-fetal interface. Data exist to implicate both excessive NK cell activation and the lack of NK cell function in reproductive disorders. This suggests there is a delicate balance that must be maintained where fetal-derived trophoblasts and maternal decidual cells work together to allow proper placental development and fetal growth.

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CHAPTER 9

The Role of Corticotropin-Releasing Hormone (CRH) on Implantation and Immunotolerance of the Fetus

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Introduction

The hypothalamic neuropeptide corticotropin-releasing hormone (CRH), as well as its receptors, have been identified in several reproductive organs, including the endometrial glands, the decidualized endometrial stroma and the placental trophoblast, synctiotrophoblast and decidua.[1-9] "Reproductive" CRH is a form of "tissue" CRH (CRH found in peripheral tissues), analogous to the "immune" CRH detected in peripheral inflammatory sites.[10] "Immune" CRH possesses potent proinflammatory properties, influencing both innate and acquired immune processes. Intrauterine CRH may participate in local immune phenomena associated with embryo implantation (Table 1).

During blastocyst implantation, the maternal endometrial response to the invading embryo has characteristics of an acute, aseptic inflammatory response; yet, once implanted, the embryo suppresses this response and prevents rejection. Simultaneously, the immune system of the mother prevents a graft versus host reaction deriving from the immune system of the fetus.

The Fas receptor and its ligand (FasL) play an important role in the regulation of immune tolerance. The major function of the Fas-FasL interaction is the induction of apoptosis in activated cells carrying Fas.[11]

We have reported that embryonic trophoblast and maternal decidua cells, i.e., cells located in the interface between the fetal placenta and the maternal endometrium, produce CRH and express FasL.[12] Trophoblast cells express a cytoplasmic form of FasL, which is secreted via the release of microvesicles.[13] CRH may play a crucial role in the implantation and the anti-rejection process that protects the fetus from the maternal immune system, primarily by killing activated T cells through the Fas-FasL interaction.[12]

Intrauterine CRH

Human and rat uterus express the CRH gene.[2,3] Epithelial cells of both species are the main source of endometrial CRH, while stroma does not express it, unless it differentiates to decidua.[2,3,14,15] In addition, CRH receptors type 1 are present in both epithelial and stroma cells of human endometrium[16] and in human myometrium,[17] indicating a local effect of endometrial CRH. Inducers of CRH, such as 8-bromo-cAMP, forskolin and epidermal growth factor, stimulate the activity of the CRH promoter.[18] Estrogens and glucocorticoids inhibit and prostaglandin E_2 (PGE_2) stimulates the promoter of human CRH gene in transfected human endometrial cells, suggesting that the endometrial CRH gene is under the control of these

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Table 1. Intrauterine functions of corticotropin-releasing hormone

Intrauterine CRH	Potential Functions
Endometrial gland and decidualized stroma CRH	Decidualization Blastocyst implantation Early maternal tolerance
Blastocyst (invasive trophoblast) CRH	Blastocyst implantation Early maternal tolerance

hormones.[19] The inhibitory effect of glucocorticoids on endometrial CRH is in agreement with that found in the hypothalamus and opposite to that observed in the human placenta, indicating that the regulation of the transcription of the CRH gene is tissue-specific. The cytokines interleukin-1 (IL-1) and IL-6 stimulate the activity of the CRH promoter, an effect possibly mediated by prostaglandins, as has been described in both the hypothalamus and the placenta.[19]

In the human endometrium, a phenomenon with characteristics of an "aseptic" inflammatory reaction takes place during the differentiation of endometrial stroma to decidua. It has been shown that CRH induces the decidualization of endometrial stroma[15,20] and that it potentiates the decidualizing effect of progesterone. Progestins stimulate the expression of endometrial CRH in a cAMP-dependent manner.[21] Indeed, in stromal cells, CRH may mediate, via the CRH-R1 receptor, the cAMP-dependent part of the decidualizing effect of progesterone, an effect blocked by the cAMP inhibitor Rp-Br-cAMP. In addition to progesterone, several locally produced pro-inflammatory immune factors also exert a decidualizing effect. Thus, prostaglandins and interleukins are important members of this category of modulators. Endometrial stroma produces several inflammatory factors, including PGE_2, interleukin 1 (IL-1) and IL-6. In humans, PGE_2 enhances, while IL-1 inhibits, the decidualizing effect of progesterone.

It has been shown that CRH inhibits the production of PGE_2 by human endometrial stromal cells.[20] Therefore, endometrial CRH, in addition to its direct decidualizing effect, may also alter the decidualizing action of progesterone via its influence on the locally produced modulators, including PGE_2. In addition, CRH stimulates the production of both IL-1 and IL-6 in human endometrial stromal cells.[20] Of note, IL-1 is a principal modulator of the decidualization process, blocking the differentiation of human endometrial stromal cells induced by ovarian steroids or cAMP.[22] The stimulatory effect of CRH on stromal IL-1 suggests that the former may exert its decidualizing effect either as a principal regulator or as a modulator of progesterone, the classical decidualizing effector. Thus, a close interaction takes place within the human endometrium involving CRH, prostanoids and cytokines.

CRH Promotes Blastocyst Implantation and Early Maternal Tolerance

Early in pregnancy, the implantation sites in rat endometrium contain 3.5-fold higher concentrations of CRH compared to the interimplantation regions,[14] indicating that CRH might participate in implantation. We have shown that CRH induces the expression of apoptotic FasL on invasive extravillous trophoblast and maternal decidual cells at the fetal-maternal interface.[12] Furthermore, CRH increases the apoptosis of activated T lymphocytes through FasL induction, participating in the processes of both implantation and early pregnancy tolerance (Fig. 1). This effect of CRH is specifically mediated through CRH-R1.[12]

During implantation, the invading blastocyst secretes several inflammatory mediators, including CRH,[23] IL-1,[24] IL-6, leukemia inhibitory factor (LIF)[25] and PGE_2. Blastocyst-deriving IL-1 plays an essential role on implantation and, in mice, blockade of its effect by the specific antagonist IL-1ra inhibits implantation.[24] The effects of LIF are equally important.[25]

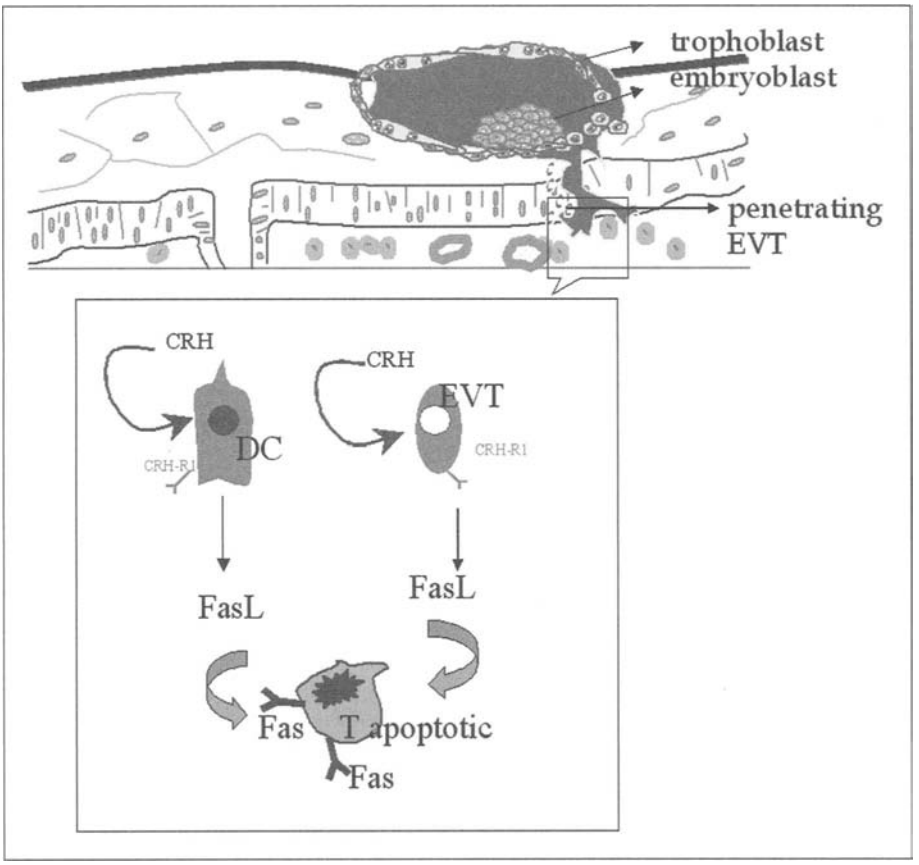

Figure 1. Role of CRH on implantation and immunotolerance of the fetus: CRH, produced locally by decidual and extravillous trophoblast cells, acts in an autocrine/paracrine fashion, through CRH-R1, to stimulate FasL expression and induce apoptosis of activated maternal T lymphocytes expressing Fas. Abbreviations: CRH: corticotropin-releasing hormone; CRH-R1: CRH receptor type 1; DC: decidual cell; EVT: extravillous trophoblast; Fas: Fas receptor; FasL: Fas ligand; T: T lymphocyte.

Preliminary data suggested that implantation could be blocked in mice by the administration of a polyclonal rabbit antiserum generated against rat or human CRH.[26] This observation is further supported by experiments in rats using antalarmin, a CRH-R1 specific antagonist. Administration of antalarmin to early pregnant rats (days 1-6 of pregnancy) results in a 70% reduction in the number of implantation sites.[12] In the rat, nidation occurs on days 4-5 of pregnancy, about 12 h after the embryo enters the uterine cavity.[27] Thus, blocking of CRH has an anti-nidation effect when it occurs at a very early stage of pregnancy. It is evident that both methods of blocking the effects of uterine CRH (antibodies or antalarmin) do not completely abolish nidation, suggesting the presence of other, redundant mechanisms supporting the implanted embryo. This is also in agreement with the fact that *CRH-* and *CRH-R1*-knock-out mice are hypofertile, but not entirely sterile.[28,29]

We have shown that CRH participates in the nidation of the fertilized egg by inhibiting local maternal immune response to the implanted embryo.[12] Our data are in agreement with previously published reports suggesting that expression of FasL by fetal extravillous trophoblast cells can induce apoptosis of activated T lymphocytes expressing the Fas receptor.[13,30,31] It

should be noted here that mice with missense or inactivating mutations of FasL gene (*gld*) can reproduce, suggesting that trophoblast FasL expression is not obligatory for maternal immunotolerance. Thus, in the absence of a functional Fas-FasL system, other mechanisms supporting maternal immunotolerance are sufficient to prevent total pregnancy failure.

It has been suggested that maternal and fetal FasL limits the migration of fetal cytotrophoblast cells into maternal tissue and vice versa.[11] Our data strengthen this hypothesis, suggesting that locally produced CRH at the fetal-maternal interface regulates FasL production and affects the invasion process through a local auto/paracrine regulatory loop of cytotrophoblast cells.

If CRH-R1 blockade by antalarmin and other compounds prevents implantation, by reducing the inflammatory-like reaction of the endometrium to the invading blastocyst, they might represent a new class of nonsteroidal inhibitors of pregnancy at its very early stages. Given the promising future of CRH antagonists in the therapy of depression and anxiety disorders,[1] their ability to cause hypofertility or early miscarriages should be seriously considered. Neverthelss, in rats, administration of antalarmin after gestation day 5, and until the end of pregnancy, did not affect the embryos, suggesting that other than CRH-mediated FasL expression mechanisms occur in mid- and late-gestation.[12] Therefore, the lack of an abortifacient or fetotoxic effect in mid- and late gestation suggests that CRH antagonists could be used to protect the fetus from maternal stress and/or to prevent premature labor, another potential use of this class of compounds.[1,32]

Conclusions

CRH, the principal regulator of the hypothalamic-pituitary-adrenal axis, as well as its receptors, have been identified in female reproductive organs, including the endometrial glands, decidualized endometrial stroma, and placental trophoblast, synctiotrophoblast and decidua.[1-9] We have shown that uterine CRH participates in local immune phenomena associated with early pregnancy, such as differentiation of endometrial stroma to decidua and protection of the fetus from the maternal immune system.[12] CRH induces the expression of proapoptotic FasL on invasive extravillous trophoblast and maternal decidual cells at the fetal-maternal interface.[12] Therefore, CRH induces the apoptosis of activated maternal T lymphocytes through Fas-FasL interaction, thus, participating in the processes of implantation and early fetal immunotolerance.[12]

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CHAPTER 10

Indoleamine 2,3 Dioxygenase-Dependent T Cell Suppression and Pregnancy

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Introduction

Viviparity remains an immunological paradox despite increased knowledge of immunological processes that occur during mammalian pregnancy. The maternal immune system protects both mother and fetus from invading pathogens during gestation, but also has to maintain immunological tolerance towards the fetus. Medawar proposed three ways in which this paradox might be resolved: physical segregation of maternal and fetal tissues, immunologic immaturity of fetal tissues, and immunological anergy (tolerance) of the maternal immune system towards fetal alloantigens.[1]

Many biochemical, or immunological mechanisms and distinct cell types have been proposed as potential factors to explain processes that suppress maternal anti-fetal immune responses. These include hormone and cytokine mediated processes that affect maternal T cell elimination, or functional deviation of aggressive Th1-type responses to relatively protective Th2-type responses.[2,3] Indeed, a recent report takes this issue a step further by demonstrating that regulatory (CD4+CD25+) T cells play a critical role in protecting fetal tissues from attack by nonregulatory T cells in a murine pregnancy model.[14] Other biochemical mechanisms and specific cell types implicated in promotion of successful outcomes in pregnancy include expression of inducible nitric oxide synthetase,[5] Fas-mediated destruction of maternal T cells,[6] uterine NK cells producing interferon γ,[7] granulocyte/macrophage growth factor, GM-CSF,[8] and macrophage growth factor, MCSF.[9] This list is by no means exhaustive, and several other regulatory cytokines and growth factors have been linked with anti-inflammatory, or immunosuppressive effects during pregnancy. These studies testify to the considerable complexity of molecular mechanisms and cellular processes that influence pregnancy outcomes, and may predispose to problematic pregnancies, such as spontaneous recurrent abortion syndromes and preeclampsia in women. One useful guiding concept is that regulation of the maternal inflammatory response is critical during pregnancy,[10] although it is also important to stress that many features of the maternal-fetal interface during successful pregnancies are reminiscent of inflammation. Since tissue inflammation is a complex phenomenon, potentially involving activation and modulation of multiple molecular, biochemical, and cellular processes, it is important to keep an open mind about the role and direction of specific processes in promoting successful pregnancy outcomes. This approach is also to be encouraged, because it has been difficult to assess the roles of specific mechanisms in pregnancy using experimental systems that provide unequivocal outcomes that address mechanistic hypotheses. While studies on mice with specific genetic-defects have begun to address these technical difficulties, redundant mechanisms can complicate analyses and confuse interpretation due to the ability of complex biological systems to compensate for defects.

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With these considerations in mind, the specific goal of this review is to summarize experimental evidence that supports a role for cells expressing indoleamine[2,3] dioxygenase (IDO; EC 1.13.11.42) in regulation of maternal immunity to allogeneic fetal tissues during pregnancy. Several studies in humans and in mouse models of pregnancy have provided experimental evidence supporting this hypothesis.[11-15] Indeed, the first suggestion that IDO might suppress maternal T cell-mediated immunity to fetal tissues came from a seminal study in which pregnant mice exposed to a pharmacologic inhibitor of IDO activity, 1-methyl-tryptophan (1mT) had significantly decreased rates of successful allogeneic pregnancies, while the effects of 1mT exposure on syngeneic pregnancies were not significant.[11,12] In the following sections, we will summarize recent research on the role of IDO expression on regulation of T cell responses, and discuss how the knowledge gained might explain the role of IDO-dependent T cell suppression during pregnancy.

Indoleamine 2,3 Dioxygenase (IDO)

IDO is a monomeric, evolutionary conserved, intracellular heme-containing enzyme that catalyzes the first, and rate-limiting step in oxidative tryptophan catabolism along the kynurenine pathway.[16] IDO-dependent tryptophan catabolism is not detectable in most tissues of healthy mice, especially when they are gnotobiotic and housed in pathogen-free facilities. IDO expression is enhanced by infection and inflammation, due to production of interferons and other cytokines that induce IDO gene expression. However, IDO is expressed constitutively at high levels in proximal male epididymis, and at the maternal-fetal interface during pregnancy in mice and humans.[11,15,17-20] Indeed, IDO activity was decreased in human placental tissues from preeclamptic pregnancies,[21,22] suggesting that IDO may help condition the inflammatory milieu at the maternal-fetal interface to decrease the risk of preeclampsia.

IDO-Dependent T-Cell Suppression by Specific Subsets of Dendritic Cells

A role for IDO expressing cells in suppression of T cell proliferation was demonstrated initially by Munn and colleagues who showed that human macrophages expressing IDO blocked in vitro T-cell proliferation by catabolizing tryptophan.[23] Subsequently, subsets of human DCs derived from cultured blood monocytes were also shown to express IDO, and suppressed proliferation of T cells in vitro due to tryptophan catabolism.[24,25] Human DCs also expressed subset-specific surface markers (CD123, CCR6), which correlated with IDO expression. This implies that the ability to express IDO is a characteristic feature of specific subsets of DCs with distinct developmental origins, though this speculative notion needs to be investigated further.

In mice, IDO expression is also associated with specific DC (CD11c+) subsets. Murine DC subsets expressing CD8α('lymphoid' DCs) suppressed delayed-type hypersensitivity (DTH) responses to tumor-associated peptide antigens displayed by CD8α- ('classic' myeloid) DCs , unless they were incubated with IDO inhibitor before injection into mice.[26-28] However, studies by Fallarino and colleagues revealed that murine macrophages and CD8α- and CD8α+ DC subsets expressed IDO protein, but that functional IDO enzyme activity was an exclusive property of CD8α+ DC subsets.[29] This suggests that additional biochemical mechanisms influence IDO enzyme activity in certain cell types. For example, IDO activity is affected by hemin availability, oxidation potentials, and nitric oxide,[30-32] and many immune response modifiers, such as cytokines. Mitogens and pathogens either induce, or suppress IDO expression and/or enzyme activity in human, or rodent cells.[33-40] The mechanisms that explain how these various factors modulate IDO gene expression and enzyme activity are known in some, but not all cases.

A recent breakthrough provided an important new insight into how IDO expression is regulated in human and mouse DCs and how the IDO mechanism modulates T cell responses under natural conditions. Grohmann and colleagues discovered that IDO inhibitor abrogated a component of the immunomodulatory effects of soluble CTLA4 (CTLA4-Ig), which is an

effective inhibitor of tissue allograft rejection in many murine transplant models, and which is showing considerable promise as an immunosuppressive drug in ongoing clinical trials in patients with autoimmune diseases.[41] Furthermore, CTLA4-Ig selectively induced IDO expression in specific subsets of splenic DCs, which blocked elicitation of T cell mediated responses in vivo.[42] These studies demonstrated that the immunomodulatory effects of CTLA4-Ig may, at least in part, be due to the ability of this reagent to up-regulate IDO expression in certain subsets of DCs via ligation of B7 molecules, which were thought to function as ligands for costimulatory molecules such as CD28 on T cells. A recent study by Munn and colleagues revealed that monoclonal antibodies to B7.1 (CD80) and B7.2 (CD86) also induced IDO enzyme activity in human CD123+ DCs, but only if antibodies to both molecules were present.[43] This provides additional support for the notion that B7 ligation in some subsets of human and mouse DCs signals IDO up-regulation, and causes them to acquire potent T cell regulatory functions. Up-regulation of IDO expression in response to artificial immune response modifiers that bind to B7 molecules may have a natural counterpart. Cells expressing surface CTLA4, such as CD4+CD25+ regulatory T cells (Tregs), may suppress responses by naïve T cells by modifying the functional properties of DCs displaying antigen.[44] Fallarino and colleagues provided support for this notion by demonstrating that transfected human Jurkat T cells expressing surface CTLA4, and physiologic murine (CTLA4+) Tregs enhanced tryptophan catabolism when cultured with murine CD11c+ DCs.[45] Interestingly, soluble CD200 (CD200-Fc), which binds to the CD200 receptor expressed on murine macrophages and γδ T cells, suppressed the rate of abortion in a murine model of spontaneous recurrent pregnancy failure, and induced IDO expression in splenic APCs that suppressed in vitro T cell proliferation.[46]

Collectively, these data suggest that CTLA4 and CD200 can cross-link cognate receptors on certain hematopoietic cell types, which suppress T cell mediated immunity by up-regulating IDO expression and enzyme activity. Presumably, similar mechanisms explain how cells expressing IDO at the maternal-fetal interface, or in lymphoid tissues draining the uterus, inhibit maternal T cell responses to fetal tissue alloantigens during gestation.

IDO Expression at the Maternal-Fetal Interface

The maternal-fetal interface is the initial point of contact between maternal immune cells and fetal cells expressing antigens that are foreign from the perspective of the maternal immune system. Thus, identifying cells that express IDO at the maternal-fetal interface is a key issue that may provide clues to the mechanism of IDO-dependent inhibition of maternal T cell responses to fetal alloantigens. As mentioned previously, several studies have focused on identifying IDO+ cells at the maternal-fetal interface in humans and mice. In humans, extravillous syncytial trophoblast cells express IDO.[17-19,25,47] Contradictory results were reported from studies using different antibody preparations to identify murine IDO+ cells at the maternal-fetal interface.[15,20] In the earlier study, antibody stained cells were located in stromal cells of the decidua basalis and metrial gland, while no staining was detected in murine trophoblastic tissues. In the second study, a polyclonal rabbit antibody against synthetic peptides bound specifically, and exclusively to primary trophoblastic giant cells (TGCs) at mid-gestation (Fig. 1). Importantly, this antibody preparation did not stain TGCs at the maternal-fetal interface when pregnant females, and their mating partners carried defective IDO alleles (IDO-KO), generated by homologous recombination in embryonic stem cell lines, indicating that TGC staining in placental tissues from normal (IDO-WT) mice was specific for IDO.

Primary TGCs develop from the mural trophoblast.[48] They undergo significant enlargement, and massive increases in DNA content, which can exceed 1000 times the haploid DNA content through endoreduplication.[49] The appearance of primary TGCs coincides with blastocyst implantation, and TGCs reach maximum size and number at day 12 of gestation in mice. Hemochorial placentation in mice allows close contact between maternal blood cells and primary TGCs inside an interlocking series of spaces that enclose circulating maternal blood. Importantly, fetal MHC alloantigens are expressed in fetal trophoblast cells from early stages of

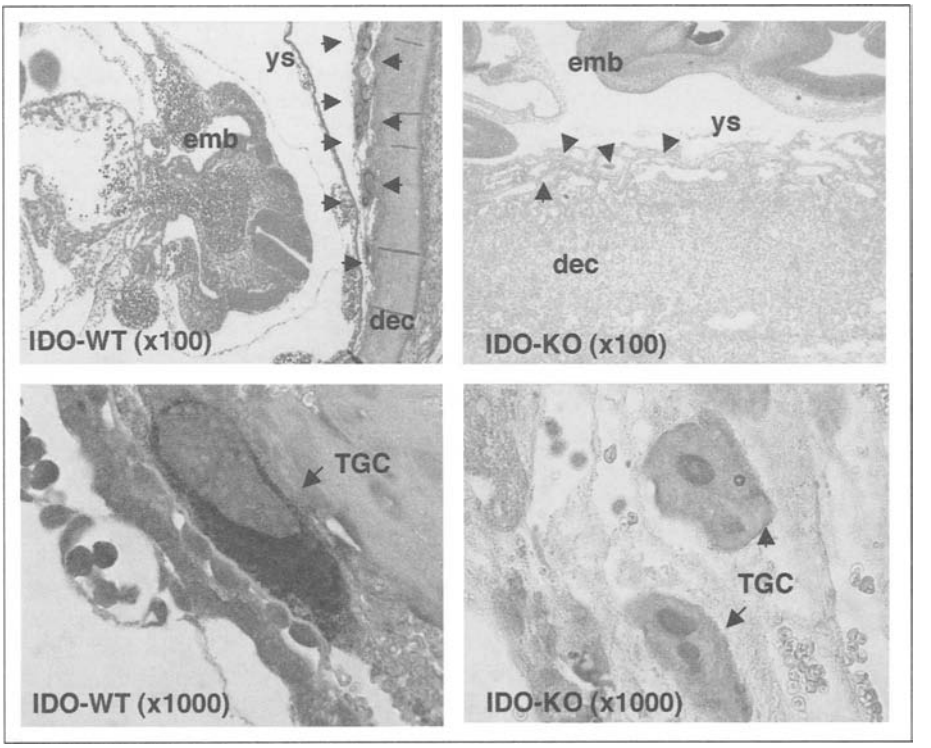

Figure 1. IDO expression is restricted to primary fetal trophoblast giant cells (TGC) at mid-gestation (E10.5) in murine pregnancy. Images are of tissue sections prepared from the maternal-fetal interface of pregnant IDO-WT, or IDO-KO mice mated with genetically identical males, and stained with a polyclonal rabbit anti-mouse IDO antibody generated against a synthetic peptide (see citation 15 for details of methods). IDO staining was specific for TGCs located at the outer border of fetal trophoblast at this point in gestation. TGCs were still present in normal numbers at the maternal-fetal interface in IDO-KO matings, but no IDO staining was detected. (Key: Emb, embryo; dec, decidua; ys, yolk sac tissues.)

gestation, but this does not compromise pregnancy success rates, even when expression is forced by transgenesis.[50-52] These and other studies to examine the effects of allogeneic pregnancy on the functional and phenotypic status of maternal T cells, suggest that maternal awareness of fetal alloantigens at the maternal-fetal interface leads to T cell suppression, and temporarily promotes T cell tolerance during pregnancy.[53,54] Thus, IDO expression at the maternal-fetal interface in humans and mice is restricted to TGCs that form the immediate physical barrier between maternal blood and fetal tissues in humans and mice. TGCs are ideally situated to influence the outcome of encounters between maternal T cells that circulate through blood spaces and cells expressing fetal alloantigens.

Extinction of Paternal IDO Gene Expression in Trophoblast Giant Cells

Analyses of the maternal-fetal interface in matings involving one normal (IDO-WT), and one IDO-KO parent revealed that maternally-inherited alleles were expressed in primary TGCs, and that paternally-inherited fetal IDO alleles were not expressed.[15] Thus, paternally-inherited alleles were selectively inactivated in fetal TGCs. Paternal allele-specific inactivation by genome imprinting has been reported for other genes.[55] Alternatively, allele-specific factors may

inactivate paternally-inherited IDO alleles selectively when primary TGCs differentiate from blastocyst trophectodermal precursor cells, when endo-reduplication amplifies the DNA content. The biological significance of extinguishing expression of paternally-inherited fetal IDO alleles in primary TGCs is not clear. One possibility is that limiting IDO activity may reduce potentially lethal consequences (for the fetus) of excessive consumption of tryptophan, which is an essential amino acid, at the maternal-fetal interface.

IDO-Dependent and IDO-Independent Regulation of Anti-Fetal T Cell Immunity

Previous studies revealed a key role for cells expressing IDO in reducing the risk that maternal T-cell-dependent, antibody-independent activation of maternal complement would cause premature fetal rejection.[12] When pregnant mice carrying allogeneic fetuses were exposed to IDO inhibitor, maternal T cells triggered complement deposition, massive local inflammation, and hemorrhagic necrosis, which caused fetal rejection. These outcomes lead to expectations that mice carrying defective IDO alleles would not bear allogeneic fetuses to term because they lacked the IDO mechanism to suppress maternal T cell immunity and complement activation. However, IDO-KO females mated with allogeneic IDO-KO males produced litters of normal size at rates comparable with IDO-WT mice.[15] These outcomes revealed that allogeneic pregnancies in IDO-KO mice were successful, even though IDO was not expressed during gestation. These data can be reconciled with previous studies in which pregnant IDO-WT mice rejected fetal allografts when exposed to IDO inhibitor,[11,56] in two ways. First, IDO inhibitor used in previous experiments caused fetal rejection by promoting IDO-independent processes. Second, IDO-independent immunosuppressive mechanisms that are normally redundant in IDO-WT mice, prevented fetal allograft rejection in IDO-KO mice, which represented an adaptation to the complete loss of the IDO mechanism in these genetically-manipulated mice. Since IDO inhibitor exposure did not increase fetal loss rates significantly in IDO-WT females carrying syngeneic pregnancies,[11] or in IDO-KO mice carrying allogeneic pregnancies,[15] the second explanation is more likely to address the apparent inconsistencies in experimental outcomes. Indeed, loss of the potent abortifacient effects of IDO inhibitor in pregnant IDO-KO mice confirms that IDO was the relevant pharmacologic target of 1mT used in the original study with IDO-WT mice, and that the IDO mechanism is the dominant immunosuppressive process that protects fetal tissues from maternal immunity during murine gestation.

IDO-independent immunosuppressive mechanisms that could compensate for the loss of the IDO mechanism in IDO-KO mice include all the processes listed in the introduction to this review (section 1). In addition, enhanced expression and activity of a second tryptophan catabolizing enzyme possessed by mammals, tryptophan dioxygenase (TDO, EC 1.13.11.11) could also help protect fetal allografts in IDO-KO mice. TDO has a completely different pattern of expression to IDO in that it is expressed at high levels in liver where expression levels are increased in response to dietary intake. Liver TDO is thought to regulate serum tryptophan levels by degrading excess tryptophan taken up from dietary sources. TDO gene transcripts have been detected in maternal-fetal interface tissues in pregnant mice[57,58] though evidence that TDO enzyme activity manifests in these tissues has not been reported. However, increased TDO activity in maternal-fetal tissues would provide an alternative compensatory mechanism in IDO-KO mice, since 1mT does not inhibit liver TDO activity.[58]

Summary

Experimental evidence from studies on human and murine DCs shows that IDO-dependent T cell suppression is a natural immunosuppressive mechanism, although the biological role of the IDO mechanism in regulating adaptive immunity is still under active investigation. IDO expression at the maternal-fetal interface in pregnant mice is limited to specific cell types of fetal origin, primary trophoblast giant cells, whose location at the extreme leading edge of the invasive trophoblast provides them with an ideal opportunity to modulate maternal T

cell-mediated immune responses to fetal alloantigens encoded by paternally-inherited major, and minor histocompatibility genes. However, it is possible that critical interactions that suppress maternal T cell immunity take place in maternal lymphoid tissues draining the uterus. Extinction of paternally-inherited IDO fetal gene expression may be a genetic response to limit IDO activity at the maternal-fetal interface. The IDO mechanism is not the only mechanism that can protect fetal tissues from destruction by T cell mediated immune responses stimulated by fetal alloantigens. Alternative IDO-independent immunosuppressive mechanisms compensate for genetic ablation of IDO expression in mice, showing that the IDO mechanism is the dominant process that protects fetal allografts from maternal immunity in wild-type (IDO-WT) mice, and that normally redundant, or sub-dominant mechanisms compensate for loss of the IDO mechanism.

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CHAPTER 11

Leukemia Inhibitory Factor in Reproduction

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Abstract

Objective

To describe the clinical findings and implications of leukemia inhibitory factor (LIF) in human reproduction. Leukemia inhibitory factor (LIF) is a pleiotropic cytokine of the interleukin-6 family and has different biological actions in various tissue systems. Although named for its ability to inhibit proliferation of a myeloid leukemic cell line by inducing differentiation, it also regulates the growth and differentiation of embryonic stem cells, primordial germ cells, peripheral neurons, osteoblasts, adipocytes, and endothelial cells. LIF is crucial for successful implantation of the embryo in mice. Currently, there is an accumulation of data about the role of LIF in reproduction.

Design

This review of literature and our studies focuses on the expression, regulation, and effects of LIF in endometrium, fallopian tube, and ovarian follicle and its role on overall reproduction.

Results

Human endometrium expresses LIF in a menstrual cycle dependent manner. Maximal expression is observed between days 19 to 25 of the menstrual cycle, coinciding with the time of implantation. Various cytokines and growth factors induce endometrial LIF expression in vitro. LIF receptor is expressed in endometrial tissue throughout the menstrual cycle and on human blastocysts in a stage-dependent manner. Affecting the trophoblast differentiation pathway towards adhesive phenotype, LIF plays a role in implantation. LIF is also expressed and secreted by the epithelial cells of the fallopian tube. Its increased expression in the tubal stromal cell cultures by the inflammatory cytokines suggests a link between salpingitis and ectopic implantation in the tube. The rising follicular fluid LIF level around the time of ovulation indicates that LIF may play a role in ovulatory events, early embryonic development, and implantation. level occurring at the time of implantation. LIF is also detected in uterine flushing, and its level is significantly lower in women with unexplained infertility. Likewise, endometrial explants derived from women with unexplained infertility showed reduced levels of LIF secretion.

Conclusions

There is growing evidence that LIF may be one of the entities that play a role in implantation, in the establishment of pregnancy, in overall human reproduction.

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Introduction

Leukemia inhibitory factor (LIF) is a pleiotropic cytokine of the interleukin (IL)-6 family with a remarkable range of biological actions in various tissue systems.[1,2] It is a highly glycosylated single chain polypeptide with a molecular weight of 20 kDa and was first described as a factor that induces the differentiation of mouse myeloid leukemic M1 cells into macrophages.[3] Glycosylation varies according to the tissue source but most often results in a glycosylated molecule with a weight of 32-67 kDa and a charge, pI: 8.6-9.2.[4] There are six cysteine residues in the murine, human, and ovine LIF and seven in the rat LIF. Disulfide bridging of these cysteines is necessary for biological activity. The number of potential N-linked glycosylation sites ranges in different species from seven to eight. The carbohydrate part of the molecule does not take part in receptor binding and is not necessary for the biological action of the molecule either in vitro or in vivo.

Each mammalian species possesses its own LIF, and the sequences of mouse, human, sheep, pig, and rat LIF have been determined.[5] Leukemia inhibitory factor is encoded by a single gene located in the mouse on chromosome 11 and in man on chromosome 22q12.[6] Both murine and human LIF have been cloned and the complete nucleotide sequence for their genes is 8.7 and 7.6 kilobase (kb), respectively.[7] Both genes are composed of three exons, two introns, and an unusually long (3.2 kb) 3'-untranslated region.[7] The LIF transcript is 4.2 kb in length. The murine and human clones that have been isolated reveal a sequence with 179 amino acid residues for the mature protein.[8,9] The homology of murine and human LIF is 79% at the protein level.[9]

Leukemia inhibitory factor is a member of a family of ligands that includes IL-6, oncostatin M, ciliary neurotrophic factor, and the recently identified cardiotrophin-1.[10,11] These factors exhibit functional redundancy since they have similar effects in a variety of biological systems, including the inhibition of embryonic stem cell differentiation in vivo and the induction of acute-phase protein synthesis in hepatocytes.[12] They also have structural similarities and all interact with the shared signal transducing glycoprotein receptor component, gp130. With the exception of IL-6, they can interact with the LIF receptor β (LIFR-β), the principal transmembrane protein that binds LIF.[13] The LIFR-β chain binds LIF specifically, but with relatively low affinity: it is only when the gp130 chain binds to LIF complexed with LIFR-β that a high-affinity receptor complex is formed and receptor activation occurs.[14] No detectable receptors for LIF are present on erythroid, granulocytic, eosinophilic, or mast cells. The majority of lymphocytes in the bone marrow, spleen, and thymus and monocytes, macrophages, and megakaryocytes exhibit LIF receptors.[15] Hepatic parenchymal cells, sensory and autonomic neurons, endothelial cells, osteoblasts, fibroblasts, adipocytes, inner layer cells of adrenal cortex, Leydig cells, embryonic stem cells, syncytial trophoblasts, blastocysts,[16] and endometrial cells are some of the nonhemopoietic cells that convey LIF receptors.[4,17,18]

Cell types known to produce LIF include fibroblasts,[19] T lymphocytes,[3] monocytes and macrophages,[20] bone marrow stromal cells,[21] osteoblasts,[22] astrocytes,[23] embryonic blastocyst cells.[24] Recently, endometrial epithelial and stromal cells,[25] ovarian granulosa-theca and stromal cells,[26] and fallopian tube epithelial and stromal cells[27] have been added to this list. Krebs ascites tumor cells, bladder carcinoma cells, and melanoma cells are some of the neoplastic cells that produce LIF.[22] Some of the signals inducing LIF transcription include endotoxin, T lymphocyte mitogens, phorbol esters, IL-1α and β, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α, platelet derived growth factor (PDGF), and epidermal growth factor (EGF).[22,25]

Leukemia inhibitory factor has multiple effects on different organ systems. It may induce differentiation and inhibit proliferation or stimulate proliferation without affecting differentiation depending on the cells analyzed.[3,8] An increase in erythroid and megakaryocytic elements and a decrease in lymphocyte size are its in vivo effects in mice.[28] Leukemia inhibitory factor inhibits in vitro differentiation of totipotent mouse embryonic stem cells without affecting their proliferation.[29] It stimulates bone remodeling[29] and inhibits lipoprotein lipase in

vivo, mediating, in part, cachexia.[30] In hepatocytes, LIF can stimulate acute-phase protein synthesis.[31] It also regulates growth and differentiation of peripheral neurons[32,33] and endothelial cells.[34] Extensive work in animal models evaluating the role of LIF in mammalian reproduction demonstrated the expression and localization of LIF in uterine tissue,[35,36] the role of LIF in the regulation and preimplantation development of the mammalian embryo,[37,38] and its importance in implantation and early pregnancy.[39-41]

LIF in Endometrium

Implantation is arguably the most critical stage in the establishment of pregnancy. It is the process by which the blastocyst becomes intimately connected with the maternal endometrium i.e., decidua. The independently developing preimplantation blastocyst then becomes dependent on the maternal environment for its continued development. Successful implantation is most likely a function of both embryonic and maternal processes. In humans, it is estimated that 30 to 70% of conceptuses are lost before or at the time of implantation.[42] Of these losses, about half are probably a consequence of genetic defects in the conceptus;[42] others are a result of defective endometrial receptivity. Finally, despite a normal embryo and adequate endometrial receptivity, altered interaction between the two may lead to failure of implantation.

Successful implantation depends upon this complex interaction between the developing blastocyst and the endometrium. It is known that for human implantation to occur, trophoblasts must attach to the endometrial surface epithelium. Then, trophoblasts interdigitate between the endometrial cells, travel through the basement membrane, and ultimately invade the maternal spiral arteries.[43] In the human, it is suggested that implantation period begins about LH day +6 and is complete by LH +10.[44] Human blastocysts remain free in the uterine lumen until day LH +5 and hatch by day LH +6. Apposition, or orientation of the blastocyst within the lumen of the uterus, starts on day LH +6. Adhesion of the blastocyst is a progressive phenomenon that tightens the embryo to the luminal epithelium. Invasion is a self-controlled proteolytic process that allows the embryonic trophoblast to penetrate deep into the maternal decidua invading the endometrial spiral arteries.

Understanding the molecular factors involved in each phase of the implantation process is critical for comprehending the mechanisms that control reproduction. Successful implantation requires a progesterone primed uterus, however, another group of autocrine/paracrine molecules, namely cytokines, adhesion molecules, and invasive proteinases also play an essential role in this process.[45] Growth factors and cytokines (especially, colony stimulating factor (CSF),[46] LIF,[47] and IL-1[48-50]) and their specific receptors are adequately distributed throughout the endometrium and blastocyst. They may also serve as the link in the regulation of molecules, i.e., adhesion molecules that provide the physical contact between the developing embryo and the uterus. All these factors, in conjunction with steroid hormones, constitute maternal-embryonic communication.

Studies on human implantation have shown many similarities to implantation in the mouse. In the mice model, LIF has been shown to be one of the essential cytokines for implantation.[47] In the murine uterus, Bhatt et al found that LIF was maximally expressed 4 days after fertilization, which is the day of implantation.[35] Following studies by Stewart et al proved the functional significance of LIF during murine implantation.[47] They found that transgenic mice lacking LIF could produce normal embryos, but the embryos failed to implant. When embryos from LIF-deficient mice were transferred to the uteri of wild-type mice, normal implantation occurred. The implantation blockade was partially corrected with the infusion of LIF into the peritoneal cavity of LIF-deficient mice, suggesting that LIF produced by endometrium is critical for murine implantation.

Since LIF is expressed in the human endometrium in a menstrual cycle dependent manner, a role for LIF in human implantation is possible.[16,25,51-53] We found the maximal expression (nearly four- to five-fold above baseline) between days 19 and 25 of the menstrual cycle, coinciding with the time of human implantation.[25] LIF receptor mRNA expression has been

found on human oocytes and blastocysts in a stage-dependent manner while LIF protein could not be detected by immunohistochemistry at any stage examined.[16,18,54,55] It seems likely that LIF is important for early uterine-blastocyst communication in the human, as it is in the mouse. While mouse and human implantation differ in that human implantation involves invasive trophoblasts and mouse implantation does not, a common feature in the implantation processes is the necessity for tight attachment of the placenta to the endometrial lining.

The patterns of LIF expression in the human and mouse endometrium differ slightly. In the mouse, two peaks of expression are detected, the first occurring at ovulation and the second around the onset of implantation. Both bursts of expression last nearly 24 hours.[35,36] The cellular localization of the ovulatory peak has not been determined, whereas the peak at implantation is restricted to the epithelium of endometrial glands. In the human, there is no evidence for a peak of expression occurring around the time of ovulation. Human LIF expression occurs in the luminal epithelium, as well as in the glands, whereas murine LIF expression is restricted to the glands.[18] In both species, if pregnancy occurs, LIF expression in the endometrium persists in the luminal and glandular epithelium. In the mouse, it is unlikely that there is an absolute requirement for the ovulatory burst of endometrial LIF expression for pregnancy, since LIF-deficient mice can ovulate and form viable blastocysts. Implantation of the embryos can be induced in the LIF-deficient mice if an exogenous source of LIF is provided on day 3 of gestation, indicating that LIF expression at ovulation is not required for preimplantation development or embryo implantation.[47] Expression of LIFR-β is restricted to the luminal epithelium, while expression of gp130 occurs in both the luminal and glandular epithelium.[18] Neither of these two receptor components appears to be expressed in the stromal cells. The patterns of expression of LIF and its receptor strongly suggest that LIF does not directly affect the stroma but acts on the luminal epithelium, possibly through an autocrine/paracrine route.

Human endometrial LIF expression is dependent on menstrual cycle.[25] LIF is expressed throughout the cycle, but there is a striking increase in the LIF mRNA levels in the mid- and late-secretory phase. Decidual tissues of the first trimester of pregnancy also express LIF mRNA but in lower levels than the secretory phase endometrium.[25] Interleukin-1, one of the important cytokines expressed in the human endometrium throughout the menstrual cycle, especially in increased levels in the secretory phase, is a potent modulator of LIF expression in the endometrial cells in culture.[25,56] Expression of LIF mRNA in endometrial glandular cells is constitutive and is relatively unregulated in comparison to the expression in the stromal cells, which is low constitutive but highly regulated.[25] Some of the other growth factors that induce endometrial LIF production are TNF-α,[57] PDGF,[58] and TGF-β.[59] Although no direct effect of estradiol or progesterone on LIF mRNA expression or LIF protein production could be shown in cultured endometrial cells by our group,[25] steroid hormones or their metabolites may be acting by way of paracrine/autocrine factors such as cytokines and growth factors to modulate LIF expression. Hambartsoumian et al on the other hand, demonstrated that endometrial LIF production was regulated by sex hormones.[60] Progesterone administration in women without ovarian function inhibited the capacity of explants to produce LIF in culture. The inhibitory effect of progesterone on endometrial LIF production was confirmed by treatment of cultured explants with progesterone in vitro. The finding by Hambartsoumian et al[60] is in discrepancy with data reported by our group and others in normally menstruating women.[25,53] Piccinni et al have recently shown that LIF is up-regulated by IL-4 and progesterone.[61] Thus, it is highly possible that the regulation of LIF secretion in normally menstruating women is under the influence of ovarian growth factors that are absent in patients without ovarian function.[62]

Potential Role of LIF in Implantation

One mechanism for LIF action in implantation could be through control of trophoblast differentiation. Depending on the external environment, undifferentiated cytotrophoblasts can differentiate along three pathways: (1) villous syncytiotrophoblasts, (2) extravillous anchoring

trophoblasts, (3) invasive intermediate trophoblasts. There is growing evidence that growth factors and peptides may mediate these differentiation pathways.[63] Biochemical and cellular markers of the trophoblast differentiation pathway have been established. The villous syncytiotrophoblast produces hCG as well as other essential pregnancy hormones.[64] In vitro experiments have shown that cAMP,[65-67] EGF,[68] and hCG[69] direct cytotrophoblast differentiation towards a hormonally active syncytiotrophoblast phenotype and up-regulate hCG production. The second type of differentiated trophoblast is the junctional trophoblast found where the chorionic villi make contact with the extracellular matrix (ECM). These cells form the anchoring cell columns seen at the junction of the placenta and the endometrium. A specific type of fibronectin, trophouteronectin (TUN), has been identified at the trophoblast-ECM junction and is thought to be a glycoprotein responsible for attachment of anchoring trophoblasts to the endometrium.[70] Transforming growth factor-β[70,71] and LIF[72] have been shown to down-regulate hCG synthesis and up-regulate TUN secretion, stimulating trophoblast differentiation toward the anchoring phenotype. The third pathway leads to the development of invasive trophoblasts which interdigitate through the extracellular spaces of the endometrium and penetrate the maternal spiral arteries. These trophoblasts produce a variety of proteases[73-75] and protease inhibitors,[76] which may mediate invasion. Unlike hCG, progesterone is produced and secreted by cytotrophoblasts as well as syncytiotrophoblasts[77] and is produced by trophoblasts in culture.[66] Thus, progesterone secretion appears to be independent of the trophoblast differentiation pathway and subsequently of LIF.[72]

Exposure of cultured trophoblasts to LIF results in a decrease in hCG mRNA and protein with a concomitant increase in fibronectin mRNA and TUN secretion.[72] This pattern of response to LIF suggests that this cytokine, like TGF-β,[70] shifts the trophoblast differentiation pathway away from the hormonally active syncytiotrophoblast phenotype towards the anchoring junctional trophoblast phenotype.

Nachtigall et al purified human cytotrophoblasts from term placentas and cultured in two groups, with and without 10 ng/mL of LIF.[72] They measured levels of hCG (a marker of villous syncytiotrophoblast differentiation), oncofetal fibronectin (a marker of trophoblast differentiation to an anchoring phenotype) and progesterone (P) at 24, 48, 72, and 96 hours. Their results showed that LIF was not produced by the cultured trophoblasts, and no difference in DNA content or P was observed between the LIF and nonLIF groups. After 24 hours, the LIF group had significantly lower levels of hCG than the nonLIF group, with 3.5-fold less at 72 hours and 4.7-fold less at 96 hours. The amount of hCG decreased with increasing concentration of LIF from 0.01-1 ng/mL and reached a plateau at higher LIF concentrations. There was no significant difference in oncofetal fibronectin between the two groups for up to 72 hours, but at 96 hours, there was a two-fold increase in the LIF group compared with the nonLIF group. Starting at 48 hours, the ratio of flat to round cells in the trophoblasts was greater for the LIF group. These changes are characteristic of cytotrophoblast differentiation toward an anchoring extravillous phenotype, modulated by LIF.

In theory, abundance of LIF around the luminal and glandular epithelium initiates embryo attachment. Once the embryo traverses the epithelium and begins stromal invasion, its own secretion of IL-1,[56] TNFα,[57] PDGF,[58] and TGF-β[59] induces further LIF production in the surrounding endometrial stroma, which in turn may stimulate production of fibronectin by the trophoblasts and facilitates implantation.

LIF in the Human Fallopian Tube

The human fallopian tube is a conduit that has a major role in oocyte pickup, fertilization, early embryonic stem cell proliferation, and embryo transport. The fallopian tube mucosa provides an embryotrophic environment that can improve implantation rates as seen with tubal transfer[78] and tubal epithelial coculture[79] in assisted human reproduction. The importance of LIF in successful early embryo development,[80] in the prevention of embryonic stem cell differentiation, and in implantation[47] led to the investigation of LIF in the human fallopian tube.

Leukemia inhibitory factor is both expressed and secreted by the human fallopian tube epithelial cells in culture in a high constitutive pattern.[27] This high constitutive secretion of LIF may be important in supporting early pluripotent embryonic cells and may lead to greater viability of the early embryo. This is suggested by studies that have revealed that human LIF both improves the viability of ovine embryos and increases murine trophectoderm mass.[80] Moreover, marked up-regulation of LIF expression and secretion in tubal stromal cultures is seen after treatment with inflammatory cytokines and growth factors. This cytokine enhancement of LIF secretion in the fallopian tube stroma may have implications for ectopic implantation. Interestingly, human chlamydial salpingitis is associated with at least a five-fold elevation of TNF-α in the tubal fluid,[81] and infection of human tubal explants with gonococci induces more than six-fold elevation in TNF-α.[82] The enhancement of LIF in the tubal stroma may represent one mechanism linking salpingitis with ectopic implantation. Elevation of LIF in both contralateral and ipsilateral isthmic segments of a chronic tubal ectopic pregnancy further suggests that an elevation in LIF, probably resulting from chronic inflammation, may not be a result of ectopic pregnancy, but may instead itself, be a factor in predisposing the embryo to ectopic implantation.[27]

Recently, it has been shown that incubation of spermatozoa in the media with LIF results in greater sperm motility and survival rates.[83] The effect of LIF on sperm motility and survival was concentration-dependent and significantly higher after 24 and 48-h exposure, respectively. Leukemia inhibitory factor showed its maximal effect on sperm motility at a concentration of 5 ng/ml, whereas sperm survival was enhanced at higher concentrations. The maximal effect on sperm survival was reached at a concentration of 10 ng/mL. Segmental expression of LIF suggests that it may also modulate maturation and transport of spermatozoa in human fallopian tube.[27] Leukemia inhibitory factor increased sperm motility at lower concentrations than it increased sperm survival in vitro. Likewise, relatively low levels of LIF mRNA and protein in the proximal part of the fallopian tube may enhance sperm motility and progression of sperm cells towards the ampullary part, where fertilization occurs. It is possible that higher LIF expression in the ampullary part of the fallopian tube may imply that everlasting motility and longer life of the spermatozoa may be more beneficial for fertilization.[83]

LIF in Ovarian Follicle

The microenvironment of human follicles is vital for oocyte development, folliculogenesis, and timely ovulation. Numerous studies have revealed that a variety of cytokines are capable of affecting ovarian function and are implicated as regulators of gonadal steroid secretion, corpus luteum function, embryo development, and implantation.[84,85] Follicular fluid provides the environment in which oocyte maturation occurs, and affects fertilization and early embryonic development. In assisted reproductive technologies, follicular fluid has been shown to improve the in vitro development of human preimplantation embryos and pregnancy rates.[86,87]

We and others, have investigated the expression of LIF in human follicular fluid and ovarian cells.[26,88] We obtained follicular fluid and granulosa-lutein cells from 123 women undergoing IVF, from 4 women undergoing ovarian stimulation, and from 3 women undergoing laparoscopy for tubal ligation during their follicular phase. Ovarian tissue was obtained from 27 women of reproductive age undergoing hysterectomy with oophorectomy for reasons other than ovarian disease. Coskun et al collected human follicular fluid from preovulatory follicles of 24 patients undergoing IVF treatment and from immature follicles of 6 patients whose IVF treatment was stopped because of the presence of large numbers of small follicles (<10 mm in diameter).[88] Both studies demonstrated that LIF concentrations were significantly higher in preovulatory follicles than in developing follicles.[26,88]

Therefore, the concentration of LIF in the follicular fluid rises around the time of ovulation and LIF levels correlate with follicular fluid estradiol levels.[26] Both granulosa-lutein cells and ovarian stromal cells express LIF mRNA and produce the protein. Granulosa cells from preovulatory follicles produce significantly more LIF when treated with hCG in vitro, suggesting

that hCG stimulates LIF production. Modulation of LIF in these cells suggests that LIF may play a role in the physiology of ovulation, estrogen production, and early embryonic development in humans. Our finding that a significant correlation between follicular fluid LIF concentration and embryo quality in an oocyte donor group[26] further confirms this assumption. On the other hand, some others[89] reported that LIF follicular fluid concentration was not associated with successful development of IVF embryos. Thus, the influence of follicular fluid LIF on embryonic development remains controversial.

Clinical Applications of LIF

In most in vitro fertilization programs, embryos are transferred to the uterus 2-3 days after fertilization (4-8 cells) because in standard culture media the majority of embryos (75%) fail to develop beyond this stage.[90] However, there are many compelling reasons for transferring embryos at the blastocyst stage, including improved synchronization between embryo and uterus and the ability to select better quality embryos over the longer culture period. There have been a number of studies which have shown increased implantation rates after blastocyst transfer,[91] particularly in women with repeated previous implantation failures.[92] Blastocysts for these earlier studies were obtained by coculture of embryos with feeder cells (e.g., Vero cells), a technique which can more than double blastocyst formation rates.[93] However, coculture is time consuming and expensive and concerns have been expressed about the possible transfer of disease from contaminated cultures.[92] A safer and more practical approach would be to produce a defined culture medium which is able to sustain embryo development throughout the blastocyst stage.

Addition of a range of factors such as vitamins and amino acids to simple culture media significantly enhances mouse, sheep, and human blastocyst development and hatching.[92,94,95] A wide range of growth factors and cytokines such as insulin,[96] insulin-like growth factors,[97] EGF,[98] and CSF-1[99,100] stimulate mouse embryo development. Platelet-derived growth factor, TGF-α, and basic fibroblast growth factor accelerate the development of bovine embryos.[101]

Additionally, LIF has been reported to augment blastocyst formation and hatching in mouse and bovine embryos, and subsequent implantation rates in sheep.[80] Early murine embryos cultured with LIF demonstrate increased trophectoderm mass and an increased rate of hatching.[80,102] In ovine embryo experimentation, the addition of human LIF to in vitro culture media increased the number of blastomeres, decreased the rate of embryo degeneration, increased the rate of blastocyst hatching, and increased the pregnancy rate.[80]

The effect of LIF on human embryos cultured in vitro has been studied by few investigators. Dunglison et al[103] added LIF (1,000 IU/mL) to spare embryos donated by couples undergoing IVF and found that blastocyst formation was increased from 18% to 44% ($P<0.025$) and that the percentage of blastocysts of optimal quality was increased from 10% to 33% ($P<0.025$). However, no blastocysts developed beyond day 7 or hatched.

Jurisicova et al[104] split randomly 463 spare embryos in the two-pronuclei stage into five groups: in four groups, LIF was added to the standard medium at the early-cleavage stages (control group, n = 164; study group, LIF at 5 ng/mL, n = 54; LIF at 7.5 ng/mL, n = 78; LIF at 10 ng/mL, n = 87; and LIF at 20 ng/mL, n = 80). Overall, in vitro preimplantation development of human embryos was poor. In the control medium, only 28% of the embryos developed to early blastocyst stage (day 5), and 19.5% developed to expanded blastocyst stage (days 6 and 7). Addition of LIF at 5, 7.5, 10, and 20 ng/mL did not improve rates of development to early or expanded blastocyst stages. There was a slight increase in rate of blastocyst formation and expansion in both 7.5 and 10 ng/mL LIF-treated embryos (38% and 36%, respectively). However, this difference was not significant. There is a controversy between these two studies regarding the effect of LIF on embryo development and blastocyst formation, and further studies are required.

Recent clinical studies have confirmed the role of LIF in various aspects of implantation (Table 1). In the uterine flushings of fertile women, LIF is detected on day LH+7, and increases

Table 1. Leukemia inhibitory factor in reproduction

Finding	References
Endometrial LIF expression, modulation, and protein production throughout the menstrual cycle	16,25,52
The effect of LIF on human trophoblast differentiation	72
LIF expression and protein production in the human fallopian tube	27
LIF receptor in human placenta	17
LIF receptors on human oocytes, and preimplantation embryos	16,54,55
LIF expression in the human follicular fluid and ovarian cells	26, 89
Effects of LIF on sperm motility and survival rates	83
Effects of LIF on human blastocyst formation rates	80,102-104
Endometrial LIF secretion and its relationship to sonographic endometrial appearance	111
Endometrial LIF production and its presence in uterine flushings	105-107
Effects of LIF on human reproductive failure	108,109

gradually to a maximum on day LH+12. In addition, the amount of LIF in the flushings obtained from women with unexplained infertility is found to be significantly lower than in those from normal fertile women.[105] Moreover, with a cut-off point of 8.23 pg/ml for LIF level in uterine flushing, a sensitivity of 86.7% and a specificity of 100% were achieved in detecting women with idiopathic infertility compared to fertile controls,[106] suggesting this technique as a possible new diagnostic tool for impaired fertility. However, in a recent study performed on 148 IVF patients, authors were unable to show a difference in mean LIF levels of endometrial flushings between pregnant and nonpregnant women.[107]

Changes in LIF expression in the human have been associated with reproductive failure during the implantation period. In-vitro endometrial explant cultures from fertile women (n = 17) secrete significantly higher LIF concentrations during the secretory phase than the proliferative phase ($p<0.05$). In contrast, infertile women do not exhibit changes in LIF expression. Women who experienced multiple implantation failures during IVF (n = 32) demonstrated decreased LIF secretion during the secretory phase. On the other hand, fertile women secreted 2.2-fold higher LIF concentrations than infertile women during the secretory phase ($p<0.05$).[108] To investigate the prevalence of LIF gene alterations in women with unexplained infertility and with recurrent failure of implantation after IVF, 200 women (45 women with recurrent failure of implantation after IVF, 50 with unexplained infertility, and 105 fertile women as controls) were screened for LIF gene mutations.[109] It has been concluded that potentially functional mutations in the LIF gene do infrequently occur in women with unexplained infertility, playing a probable role in the etiology of infertility. However, routine screening for LIF mutations or polymorphisms in these women is not currently justified for the low prevalence of gene alterations.[109]

Another study demonstrates that early luteal phase treatment (200 mg) as well as intermittent or daily low dose (0.5 mg) treatment with mifepristone, an antiprogestin, reduces endometrial glandular LIF expression at the expected time of implantation.[110] Thus, the contraceptive potential of mifepristone in low doses may be via its inhibitory effect on endometrial LIF expression. Recently, a recombinant human LIF (rhLIF) has been developed. It is produced using *E. coli* as an expression system in a manner that makes it suitable for human clinical use. This compound (rhLIF), an aqueous solution suitable for subcutaneous administration, is currently under preclinical and clinical investigations.

Conclusion

Leukemia inhibitory factor, a pleiotropic cytokine with a remarkable range of biological actions in various tissue systems, may have a role in embryo implantation. Several cytokines and growth factors have different effects on LIF expression; meanwhile LIF may have diverse effects on integrins and adhesion molecules to affect implantation. Human endometrium expresses LIF in a menstrual cycle–dependent manner, with maximal expression coinciding with the time of potential implantation. Leukemia inhibitory factor receptor is expressed in human endometrial tissue throughout the menstrual cycle and is expressed in human embryos in a stage-dependent manner. For the embryo, LIF appears to affect the trophoblast differentiation pathway, influencing it toward the adhesive phenotype. Results obtained from uterine flushings and endometrial explants suggest that defective LIF expression may contribute to human infertility. Leukemia inhibitory factor seems to affect sperm survival rates and motility in the fallopian tube, thus, playing a role, even on fertilization. Based on reports gathered from animal and human studies, LIF appears to play an important role in implantation and in the establishment of pregnancy. A recently developed r-hLIF is planned for human clinical trials for its physiological role in leading to enhanced reproductive success. Future studies on LIF will be helpful in solving different clinical problems including the etiology and pathogenesis of ectopic pregnancy and low implantation rates in assisted reproductive technologies.

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CHAPTER 12

Characterization of Human Dendritic Cells at the Materno-Fetal Interface

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The unusual tolerance against fetal antigens is still one of the greatest miracles of pregnancy. Dealing with reproductive immunology, the question arises as to how the maternal immune system handles the foreign fetal antigens leading to that tolerance. Focussing on the various subsets of immunocompetent cells playing in concert with the human immune system, tolerance induction nowadays is often accredited to a newcomer, at present the "hottest star" for immunologists is the dendritic cell (DC).

Dendritic Cells within the Immune System

DC are a heterogeneous population of bone-marrow derived cells present in minute numbers scattered all over the body, yet they are the most potent of all antigen presenting cells (APC).[1] APC are specifically equipped to initiate and maintain immune responses, they are able to uptake and process antigens and present them to T cells. APC seed surface areas, such as the epidermal layer of the skin and the mucosal membranes as well as the interstitial spaces of solid organs, playing a "sentinel" function for the immune system.[2,3] Among these APC, bone marrow derived DC are the most potent activators of naive T lymphocyte responses.[4,5] The "life cycle" of a DC is principally characterized by the changing states of functional activity that the cells have to pass through to optimally fulfil their mission. First, in a so-called immature state, DC function as sentinels of the immune system patrolling the tissues and the blood stream.[1] Upon local tissue disturbance, for example caused by invading pathogens, resident immature DC are activated and, together with additional APC attracted by chemokines, accumulate in the inflamed tissue,[6] to pick up the pathogen's antigens. To do so, immature DC express several DC-characteristic adsorptive receptors mainly belonging to the lectin family. Some of the most important receptors are the MMR (macrophage mannose receptor; CD206) and DEC205 (CD205), which are both large type I membrane proteins.[7] Besides those two receptors, DC express several type II transmembrane proteins with a single external C-type lectin domain. One of these monolectines is DC-SIGN (**d**endritic **c**ell-**s**pecific **I**CAM- **g**rabbing **n**onintegrin, classified as CD209) which is a DC-specific adhesion receptor with high affinity for the adhesion molecules ICAM-2 and ICAM-3.[8] DC-SIGN can be found predominantly on immature DC and is capable of binding various antigens, for example the HIV protein gp120.[9-11] Following rapid internalization of exogenous antigens (by receptor and also nonreceptor mediated endocytosis) into endosomal compartments for processing of the antigen into peptides, DC initiate their maturation program and migrate from the site of inflammation via the lymph vessels into the draining lymph nodes. Concomitantly DC start upregulating their costimulatory activity such as expression of surface molecules CD40, CD80, CD86 and CD83 as well as T cell activating cytokines like IL-12 and thereby mature into potent immunostimulatory APC.[12] Recently evidence has accumulated that the fine-tuning of

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DC maturation is governed by "danger signals" the DC encounter at the local inflammatory site. Examples for "danger signals" include the inflammatory cytokines IL-1 or TNF-α, which are released by the damaged tissue itself, or products of the invading pathogen, such as endotoxins, bacterial DNA or viral double stranded RNA which activate DC via receptors of the TollR family and thereby instruct the APC to mount an adequate immune response.[13] After reaching the lymph node the final maturation of a DC is established during cognate interaction with antigen specific $CD4^+$ T helper cells resulting in the capacity to efficiently stimulate $CD8^+$ effector T cells with lytic activities. The last part of a DC's life cycle is terminating their antigen presenting and T cell stimulating activities by undergoing apoptotic cell death.

The main function DC have long been studied for, is their capacity to activate T cell responses in vivo and in vitro.[12] However, in addition to their significant stimulatory capacity, DC have an important regulatory role in the immune system, including the induction of peripheral tolerance and regulation of the types of T cell responses. In addition DC may also act as effector cells in innate immunity against microbes. These diverse functions of DC in immune regulation are now recognized to depend on the diversity of DC subsets and functional capacities resulting from maturation.[14]

Concerning the function of DC as tolerance inducing mediators, there is evidence that DC can be converted to Th2 skewing cells when treated with anti-inflammatory cytokines such as IL-10[15,16] or the glucocorticosteroid Dexamethasone.[17] In the mouse and rat model, DC isolated from Payers patches in the gut elicit Th2 responses,[18,19] whereas those from spleen induce Th1 responses.[20] There is further evidence that DC in mucosal surfaces of the gastrointestinal tract are responsible for oral tolerance of antigens.[21] This may reflect a special DC subpopulation prone to act as "tolerance inducing" APC seeding mucosal surfaces. Thus, a similar subpopulation could be responsible for maternal tolerance against fetal antigens in the mucosal decidua.

Characterization of Human Dendritic Cells in Endometrium/Decidua

The precise mechanism by which the maternal immune system deals with fetally-derived antigens during pregnancy is still unknown. Dendritic cells at the feto-maternal interface would be ideally sited for this purpose and therefore it seems very likely, that human endometrium, the uterine mucosa, is seeded with DC like other mucosal surfaces. However, nearly all studies on the immunology of pregnancy concentrated on uterine NK cells and T cells. There is still remarkably little information on dendritic cells in uterine tissue. The first report to describe the idea of antigen-presentation in the context of endometrium dates back to 1983, when Sutton et al characterized $HLA\text{-}DR^+$ cells in human placenta and decidua, the mucosa of the pregnant uterus.[22] By immunohistochemistry they found many cells irregular in shape which stained strongly for HLA-DR antigen and were widely spread in human decidua (Fig. 1). Those cells were proven to be of maternal origin by HLA-typing. Already at this early time point, when almost nobody had studied dendritic cells, the authors speculated, that these $HLA\text{-}DR^+$ cells are either macrophages or, given their stellate shape, are related to the dendritic cells "of the Steinman type". In 1984 Bulmer and Sunderland quantified $HLA\text{-}DR^+$ cells in the decidua by immunohistochemistry.[23] They found that these cells account for 40-50% of the decidual leukocytes, and were often irregular in shape and prominent around spiral arteries which are typical for the pregnant uterus. Cells of similar number, distribution and irregular morphology were found with the anti-leu-M3 antibody, a monoclonal antibody (mAb) reactive with tissue macrophages. Therefore they concluded that the $HLA\text{-}DR^+$ cells mainly belong to the macrophages. They also described occasional cells positively stained with the human thymus antigen antibody NA1/34 immediately beneath the endometrial gland endothelium and, in a few decidual tissue sections, around the spiral arteries. This antibody NA1/34 was subsequently defined as specific for CD1a, a marker for dendritic cells of the Langerhans lineage. Some years later Oksenberg et al demonstrated that human decidua harbours antigen presenting cells (APC)

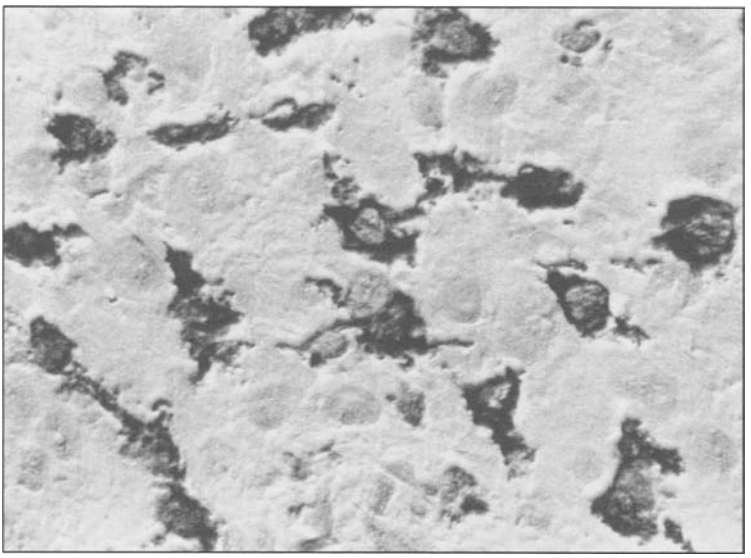

Figure 1. Immunohistochemistry of human decidua with cells with typical dendritic cell morphology (HLA-DR, purple, magnification x400). A color version of this figure is available online at http://www.eurekah.com.

that could be stained with the antibodies 63D3 (macrophages), W6/32 (HLA-class I) and 7.2 (HLA class II), and were able to induce T cell proliferation.[24] They assumed, that those decidual APC are "dendritic like Ia positive cells" and speculated that they function as the key cells in the mutual immune recognition between mother and fetus, which may result in a protective rather than destructive maternal immune response. In the later course, Kamat and Isaacson showed by immunohistochemistry, that macrophages (identified by UCHM1 and HLA-DR$^+$) were diffusely distributed in the stroma of human endometrium.[25] They also identified within the glands of the endometrium large mononuclear Na1/34$^+$ cells resembling Langerhans cells in their dendritic morphology. However, cells positive for HLA-DR, a typical marker for APC, were not seen in their study. One year later, Lessin et al performed a systematic study of HLA expression by human decidual cells throughout gestation.[26] Like Oksenberg, they found a major (21-32%) proportion of maternal decidual cells with strong positivity for HLA class I and class II molecules. In early gestation, they found class II positive cells predominantly adjacent to the syncytial trophoblast, whereas later in gestation, the class II positive cells were randomly scattered throughout the decidua. Those class II positive cells were identified as macrophages by 63D3 staining. In the same year, Dorman and Searle reported the alloantigen presenting capacity of cells (with still unknown identity) in human decidual tissue.[27] The authors speculated that the decidua may harbour different populations of APC, one of them belonging to the class II positive dendritic cell lineage.

More recently, in vitro investigations have shown conflicting data about decidual macrophages as the main cell population exerting antigen presenting properties in human decidua. Using the antibodies Ki-M7 and Ki-M8, specific for cells of the monocyte/macrophage system, Dietl et al characterized numerous leukocytes in first trimester pregnancy decidua but did not provide a more precise description of the cells.[28] In the same year, Searle et al managed to isolate decidual APC from whole-decidua cell suspensions via a plastic adherence step.[29] Adherent cells were pulsed with antigens (of fetal chorionic cells) and subjected to phytohemagglutinin (PHA)-induced allogeneic PB lymphoproliferation. In this assay, the authors could

not demonstrate human early pregnancy decidual APC mediated immunoregulation, because in all experiments decidual APC did not suppress mitogen induced T cell proliferation (as expected by the authors). In 1994, Mizuno et al desribed, that isolated decidual macrophages are able to present soluble antigens in an MHC-restricted manner but also possess some suppressive activity for the maternal immune response.[30] Thus they suggested that macrophage play a local role in the maintenance of pregnancy. Contrary to these findings, Olivares et al in 1997 described decidual stromal cells that did not express the classical macrophage marker CD14 as measured by flow cytometry, but did express HLA-DR as well as the activation markers CD80 and CD86.[31] Since these cells were potent stimulators of allogeneic T cells, the authors were in favour of professional APC seeding the human decidua. A few years later, Abraham et al were the first to characterize by electron microscopy using Zink-Iodide-Osmium fixed decidual samples, two distinct types of decidual DC.[32] One with a lobulated, heterochromatic nucleus and few mitochondria and ribosomes and a second with a round or oval euchromatic nucleus, more mitochondria, free ribosomes and pieces of rough-surfaced endoplasmatic reticulum. The concrete nature of those decidual APC was not further characterized until 2000, when it was demonstrated by our group, that human endometrium and early pregnancy decidua harbours a subpopulation of HLA-DR positive cells expressing the "classical" DC-marker CD83 with morphologic and functional features of typical mature DC, similar to those described for other mucosal surfaces.[33] During over night culture, the number of $CD83^+$ mature DC in the decidual samples increased massively implying that decidua harbours immature precursors, which could be responsible for antigen processing. Further evidence in search for the immature precursors of decidual DC, was provided by Soilleux, who detected DC-SIGN on HLA-DR positive decidual cells and described them as specialized decidual macrophages by their costaining for CD14 and the "classical" macrophage-marker CD68.[34] We then characterized the DC-SIGN positive decidual cells as precursors of immunostimulatory decidual DC by proving in vitro that isolated decidual DC-SIGN positive cells can mature into classical CD83 expressing DC with high T cell stimulatory capacity.[35] Interestingly, compared to nonpregnant endometrium, numbers of immature DC-SIGN positive DC were greatly enhanced in decidua. Whether this increase of DC-SIGN expressing cells is caused by an upregulation of DC-SIGN on CD14 positive cells or by an in situ-proliferation of DC-SIGN positive DC is not known yet. The latter hypothesis is supported by the detection of a remarkable fraction of proliferating intradecidual DC-SIGN positive cells,[35] however, a definite answer requires future investigations. Also in 2003, Gardener et al by three-colour flow cytometry identified a small (1,7% of decidual CD45 positive leukocytes) population of decidual DC expressing HLA-DR, and CD11c, a marker for myeloid DC, but none of the other classical leukocyte lineage markers such as CD3, CD14, CD16, CD19, CD20 or CD56.[36] The CD11c positive cells were generally found to be negative for DC-SIGN and had a low expression of the lectin receptor DEC205 and the costimulatory molecules CD80 and CD86, consistent with an immature state of these DC. The authors subdivided the CD11c positive decidual DC population in two subgroups related to the intensity of HLA-DR expression and discussed the $HLA\text{-}DR^{bright}$ group to be more mature. Confirmation of such a $HLA\text{-}DR^{bright}$ population of DC was given in a recent report by Miyazaki et al who could clearly demonstrate by FACS analysis, that human early pregnancy decidua contains lin^- $HLA\text{-}DR^{bright}$ DC.[37] In contrast to Gardener et al, these DR^{bright} DC displayed a full mature phenotyp with high expression of CD83 and CD86 and even higher expression of CD80 compared to DC obtained from peripheral blood. Based on some in vitro functional data the authors claimed that decidual DC may be able to regulate the Th1/Th2 balance and maintain a Th2 dominant state protective for pregnancy. In a very recent publication, Askelund et al compared the numbers of mature DC in human decidua of normal early pregnancy and recurrent missed abortion.[38] They found a significant higher number of mature DC in decidual tissue from abortions and speculated, that mature DC may play a role in the pathophysiology of some cases of recurrent abortions.

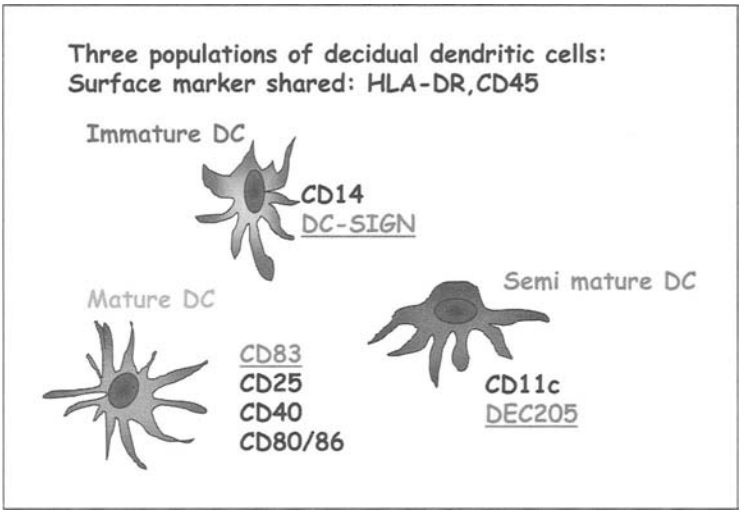

Figure 2. Cartoon of the three dendritic cell (DC) types in human decidua. All three types are characterized by the expression of CD45 and HLA-DR on their surface. In addition, the subtype specific surface marker are given on the right side of the cell with the key marker underlined.

In summary, decidua of early human pregnancy harbours at least 3 different groups of DC populations: classical mature CD83 positive DC,[33,38] immature DC-SIGN expressing DC prone to mature into potent immunostimulatory DC[34,35] as well as a very small group of DEC205 positive immature DC (Figs. 2, 3).[36]

The Functional Role of Decidual Dendritic Cells

The function of dendritic cells in the human decidua is still far from being understood. It is very likely that, as specialized APC, decidual DC do present fetal antigens (derived from the invasive trophoblasts) to the maternal immune system, but how this function of antigen presentation function relates to inducing a state of tolerance to fetal antigens is unclear. It seems possible, that the intradecidual microenvironment and cellular interactions decide whether immature DC will acquire characteristics and functions of classical antigen presenting mature DC with T activating features, or—more likely—arrest the DC in an immature or "semi mature" state which is thought to mediate tolerance induction.[39] In this respect, it is noteworthy that many of the factors described so far to promote tolerogenic DC are present in abundance in human decidua. These factors include IL-10, TGF-β and prostaglandin E2. Especially due to the significant amount of IL-10 produced at the materno-fetal interface,[40] an IL-10 mediated tolerizing mechanism seems worthwhile to pursue. In vitro, IL-10 has been shown to inhibit T cell activation by DC via preventing the induction of costimulatory molecules such as CD83 and CD86 on immature DC.[41] Decidual macrophages[42] and $CD56^{bright}$ $CD16^{-}$ NK (dNK) cells[43,44] have been found to produce high levels of IL-10. In this context, the close topographic relationship between intradecidual iDCs and NK cells is of special interest (see Fig. 4).[35,45] This intimate cell contact is probably brought about by the interaction of DC-SIGN expressed on DC and ICAM-3 which is expressed on NK cells.[35] This cell-cell contact may either induce an as yet unknown signal keeping the DC in a tolerant state and/or bring the DC in close contact with the NK cells as source of IL-10. Furthermore, the sterical situation

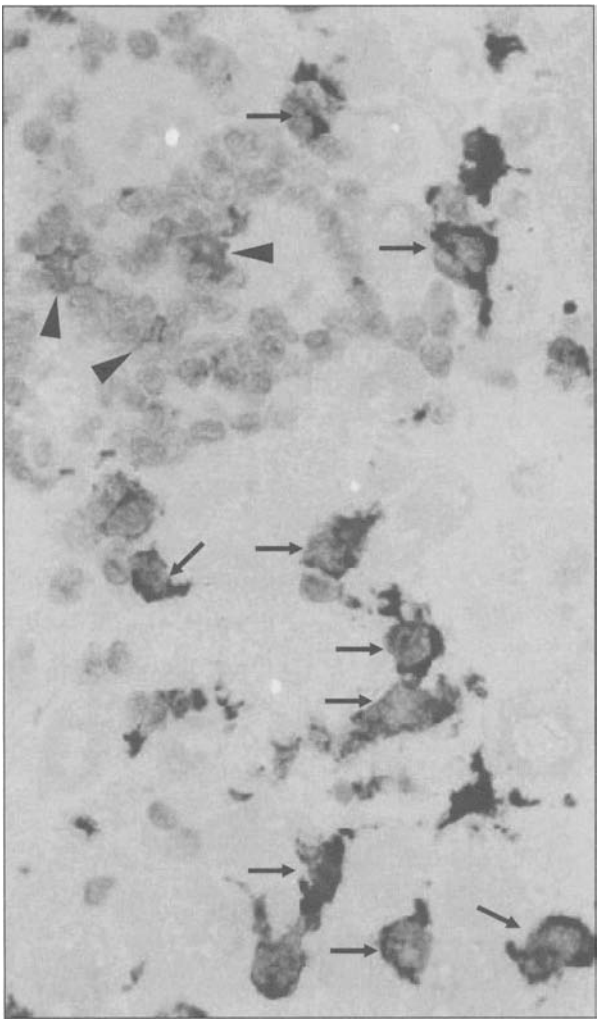

Figure 3. Immunohistochemistry on human early pregnancy decidua shows immature DC-SIGN positive immature DC (purple, arrows) scattered in the decidual stroma and attached to only few cells. On the upper left side of the picture, a cell cluster with a cluster of leukocytes surrounding three CD83 positive mature DC (green, arrowheads) is seen. Nuclei are counterstained with hematoxiline. Magnification x400. A color version of this figure is available online at http://www.eurekah.com.

resulting from intimate contacts with the dNK simply might prevent an interaction of iDCs with T-cells and the maturation of iDCs into immunostimulatory DCs. Hopefully further research on DC and their cellular companions at the materno-fetal interface will shed light on how this delicate balance between tolerance to fetal antigens and immunity to threatening agents is brought about and maintained.

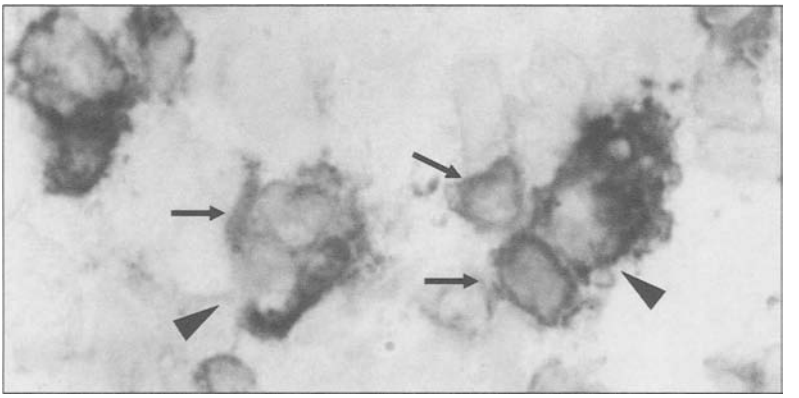

Figure 4. Immunohistochemistry clearly demonstrates the intimate contact between immature DC (purple, arrowhead) and decidual NK cells (brown, arrow) in human early pregnancy decidua. Magnification x1000. A color version of this figure is available online at http://www.eurekah.com.

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CHAPTER 13

MHC Molecules of the Preimplantation Embryo and Trophoblast

Martina Comiskey, Carol M. Warner and Danny J. Schust

Abstract

The mechanisms of protection of the allogeneic fetus from the maternal immune response during pregnancy remain mysterious more than fifty years after the paradox of maternal tolerance was first raised by Peter Medawar. Preimplantation embryos express paternal antigens early in development. After implantation, placental tissue is derived from both maternal tissue and paternal-antigen expressing fetal tissue that is in intimate association with and bathed in maternal blood. There appears to be a key role for an unusual subset of major histocompatibility complex (MHC) Class I proteins of both maternal and paternal origin in the mediation of tolerance at the maternal/fetal interface and in the control of preimplantation embryonic growth rate. This subset of MHC products is composed of two nonclassical MHC Class Ib proteins, HLA-E and HLA-G in combination with a classical MHC Class Ia protein, HLA-C. This chapter reviews the history of the discovery of the major histocompatibilty complex Class I genes and the elucidation of the biological role of the proteins encoded by these genes in the immune response and in reproduction. MHC genes have also been implicated in reproductive choice and nurturing behaviors. We hypothesize that the vertebrate immune system derived from ancestral recognition systems driven by reproductive requirements, and was later coopted for immune recognition under additional evolutionary pressures. The complex interactions of MHC Class I proteins with components of both the innate and the adaptive immune systems in the context of the preimplantation embryo and the trophoblast of early pregnancy are described in detail, as are the difficulties inherent in studying these systems. Finally, potential future directions of research and the need for new model systems to study both preimplantation embryos and the maternal/fetal placental interface are discussed.

Introduction

The acquired immune system, also known as the adaptive immune system, exhibits signature attributes of pathogen specificity and memory response. It emerged abruptly and mysteriously in the vertebrate lineage some 500 million years ago.[1] Antigen-recognizing T and B cell receptor genes, combinatorially rearranged by recombinase activating gene (RAG) enzymes, together with antigen-presenting genes of the major histocompatibility complex (MHC) apparently appeared simultaneously in jawed vertebrates. These characteristic components of the acquired immune system coexist in the earliest jawed fishes but are absent in jawless hagfish and lamprey. There is no conclusive evidence of the evolution of transitional molecules in the 50 million years between the divergence of the jawless and jawed fishes from their last common ancestor. Over time the functions of the acquired immune system in vertebrates have become

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inextricably blended with those of the innate immune system. The first goal of this review is to discuss the evolution of the MHC with respect to its role in reproduction. We will propose the hypothesis that the vertebrate immune system derived from ancestral recognition systems driven by reproductive requirements, and was later coopted for immune recognition.

The function of the MHC in graft rejection was first defined by Gorer[2] and Snell[3] in studies of the role of the mouse histocompatibility (H-2) locus in rejection of transplanted 'nonself' tissue. The equivalent human leucocyte antigen (HLA) locus was first identified by Dausset in 1958.[4] What was at first defined as a major histocompatibility genetic "locus" is now known to encompass many genes, resulting in the present day use of major histocompatibility "complex" to describe this genetic region. For many years the biological function of the MHC was unknown. The groundbreaking discovery, by Doherty and Zinkernagel,[5] that T-cells recognize peptide only when presented in the context of an MHC molecule, defined an immunological function for MHC molecules. Crystallization and X-ray diffraction studies in the laboratories of Jack Strominger and Don Wiley provided a molecular understanding of peptide presentation first by Class I MHC molecules[6] and later by Class II molecules.[7]

The MHC is a four megabase continuous region of DNA located on chromosome 6 in humans and chromosome 17 in the mouse. The complex is composed of three subregions encoding MHC Class I, Class II and Class III genes which in turn encode Class I, Class II and Class III proteins. Class I MHC genes and proteins are the main subject of this review and are discussed in detail later. Class II MHC genes are very polymorphic in human populations, and their protein products are expressed primarily on "professional" antigen-presenting cells: macrophages, dendritic cells and B-cells. Class II proteins present 13-18 amino acid peptides derived from extracellular pathogens to $CD4^+$ T helper cells and are important in the priming and maintenance of the humoral immune response. Class III MHC genes are more conserved and encode a variety of secreted proteins, some of which play a role in antigen processing, in inflammatory responses and in the complement cascade. Class II and Class III MHC genes and proteins are not discussed in detail in this review.

Class I MHC proteins are used by cells to present 8-11 amino acid intracellularly-derived peptides to $CD8^+$ T cytotoxic cells and to natural killer (NK) cells. Class I MHC molecules consist of a trimeric complex of three noncovalently bound components: the MHC Class I heavy (alpha) chain encoded in the MHC, the small antigenic peptide which is being presented to immunocytes, and $beta_2$ microglobulin (β_2m), which is encoded in chromosome 15 in humans and chromosome 2 in the mouse. Displayed peptides may be endogenous cellular peptides (self peptides) or peptides derived from intracellular pathogens (nonself peptides). Peptide-MHC Class I-β_2m complexes (pMHC) interact with the T cell receptor (TCR) on $CD8^+$ T cells of the acquired immune system, and with multiple receptors on natural killer (NK) cells of the innate immune system. MHC Class I genes are subdivided into "classical" Class Ia genes and "nonclassical" Class Ib genes. The human Class Ia genes, HLA-A, -B and -C, are highly polymorphic and are expressed on the surface of almost all nucleated cells. HLA-A and –B genetic loci were first identified by van Rood using human pregnancy serum containing alloantibodies against fetally expressed paternal histocompatibility antigens;[8] his discovery led to significant advances in kidney transplantation because these reagents could be used to tissue-type donors and recipients. A decade later the third locus, HLA-C, was identified. These products are notable for lower levels of expression at the cell surface than other class Ia counterparts.[9] Nonclassical Class Ib genes HLA-E, -F and -G were initially defined in comparison to Class Ia genes by reduced polymorphism, restricted tissue expression and unknown function. Class Ib genes and their products remain somewhat enigmatic, but recent studies have shown that they can exhibit polymorphism and either tissue restriction or more ubiquitous expression.[10]

Polymorphism in MHC Class I genes is most evident in their peptide binding region. Such polymorphism is generated by amino acid-altering mutations in the MHC genes and is sustained by balancing selection during evolution. Heterozygosity of MHC alleles in an

individual is believed to improve immune surveillance by increasing the array of peptides that can be presented to the immune system, thus enhancing protection from disease. There are intriguing data suggesting that MHC Class I genes also play a role in mate selection and other behaviors that favor increased MHC heterozygosity in offspring.[11] MHC genes are closely linked on the chromosome and alleles tend to be inherited in sets or haplotypes, one from each parental chromosome. The MHC alleles are codominantly expressed, so each individual expresses both paternal and maternal MHC gene products. In the case of Class I proteins, an individual is self-tolerant to endogenously presented peptides because of an essential and rigorous process of negative and positive selection occurring in the thymus, supplemented by peripheral mechanisms inducing tolerance or anergy in potentially autoreactive T cells.[12]

In 1953 Peter Medawar first raised the paradox of maternal tolerance to the paternal MHC in the fetus during pregnancy,[13] but to this day mechanisms of protection of the fetus from the maternal immune response are not fully understood (see Introduction). Of particular interest to this review is whether or not Class I MHC proteins are expressed in preimplantation embryos and in the trophoblast. The interactions between Class Ia and Class Ib molecules and among all Class I molecules and components of the adaptive and innate immune systems during early pregnancy are complex, redundant and as yet poorly understood. The system exhibits many inherent qualities that promote slow progress in research leading to an improved understanding of the role of MHC Class I molecules in early pregnancy. For instance, in humans there are technical difficulties in obtaining samples during temporally and spatially restricted developmental stages of pregnancy. The unique extent of placental invasion in humans makes comparison with other species of somewhat limited relevance. Information on MHC Class I expression in human embryos is sparse given the moratorium on federal funding of such work in the United States. Therefore reliance on animal models has been mandatory, even though the results may not be fully applicable to humans. The second goal of this review is to discuss MHC Class I protein expression and possible function in preimplantation embryos and in the trophoblast, with respect to protection of early embryos from rejection by the maternal immune system. The main emphasis will be on human embryos, with some reference to data from animal models where such comparisons are informative.

Evolution of the MHC

The dearth of organisms providing clues to the transitional development of the MHC during the divergence of the jawless and jawed fishes makes discussion of the selective pressures driving development somewhat speculative. There is indirect evidence for the existence of a MHC-like molecule in a colonial invertebrate urochordate. *Botryllus schlosseri*, a tunicate sea squirt, expresses a gene coding for a protein remarkably similar to vertebrate CD94, a receptor expressed in natural killer cells which binds MHC Class I in mice and humans.[14] *Botryllus* colonies that meet or are placed in contact with each other will either fuse together or develop cytotoxic lesions in an allorecognition reaction. This allorecognition is mediated by a single highly polymorphic and codominantly expressed fusibility/histocompatibility (Fu/HC) locus. Colonies that share a Fu/HC allele will fuse forming a chimera with a joint vascular system, while colonies not sharing an allele will undergo rejection. Fu/HC loci are not orthologous to vertebrate MHC loci but clearly exhibit similar characteristics. Fu/HC may be an ancestral MHC molecule, or both molecules could have evolved from a common ancestor. It is not yet known if the *Botryllus* CD94 homolog interacts with Fu/HC proteins, but it is upregulated during allorecognition responses and was identified in a differential display PCR screen comparing RNA extracted from isolated colonies with RNA from colonies undergoing allorecognition reactions.[14]

Gametogenesis involving circulating germ-line progenitor cells can occur in *Botryllus* colonies after fusion, and germ cell 'parasitism' can occur in fused colonies. In some cases one colony can be completely resorbed by the other, yet the surviving germ cells may have derived from the resorbed colony. The maintenance of a highly polymorphic Fu/HC system combined

with fusibility only with colonies sharing an allele ensures that parasitic germ cells requiring somatic resources in a fused colony at least come from a sibling colony.[15] Vertebrates do not undergo natural transplantation processes, with the exception of pregnancy, where the semi-allogeneic fetus can be considered in that light. The vertebrate immune system may thus have derived from ancestral recognition systems driven by reproductive requirements, and later modified under additional evolutionary pressures to evade pathogens.

Gene families characteristic of the human MHC-linked paralogy region (so called MHC anchor genes) appear to have been assembled and linked in the cephalochordate Amphioxus prior to the origin of vertebrates and in the absence of adaptive immune system genes.[16,17] These conserved anchor genes can be considered a "proto-MHC" present in Amphioxus, which is believed to represent the ancestral genome before vertebrate tetraploidization.[10] This framework hypothesis[18] suggests that the nonconserved Class I genes then expanded in permissive sites within the highly conserved anchoring framework genes of the MHC. It is clear that, at least in mammals, orthology is maintained only by the nonClass I MHC genes in the MHC complex.[10]

MHC and Reproductive Behavior

MHC Class Ia genes play an unequivocally central role in the immune response, but have also been shown to influence mate selection resulting in increased MHC heterozygosity of progeny. MHC-driven mate choice based on odor cues has been documented in mice,[19] and more controversially in humans.[20] There are three extant and not mutually exclusive hypotheses for MHC-dependent mating preference: augmented immune surveillance by increasing the array of peptides that can be presented (heterozygote advantage), improved defense against fast-evolving parasites (the Red Queen hypothesis), and avoidance of inbreeding.[11] The relationship between MHC genetic composition and behavior is complex, involving selection for kin recognition in addition to MHC-disassortative mate choice. Female mice, which preferentially select mates with MHC alleles different from their own, will nurse each other's pups in communal nests but appear to prefer nesting partners sharing their MHC alleles.[21] MHC-based odortypes also aid in reciprocal recognition by mothers and pups, even if the pups have been cross-fostered with an MHC-dissimilar female.[22] Intriguingly, subsequent MHC-based mate selection preferences can be altered by cross-fostering pups,[23] indicating that early learning experiences of the MHC environment are important for subsequent inbreeding avoidance as well as maternal nurturing behavior.

Odor detection of classical MHC genes provides chemical cues for recognition, evoking neural activity in the main olfactory bulb (MOB) of the mouse brain and resulting in measured behavioral responses. Indeed, a cluster of polymorphic olfactory receptor genes has been identified at the distal end of the mouse MHC and shares a history of coduplication with MHC genes.[24] The MOB recognizes volatile chemical cues. Neural responses to volatile cues are seen even in the absence of the vomeronasal organ (VNO) that detects nonvolatile pheromones and activates neurons in the accessory olfactory bulb (AOB).[25]

Mammalian pheromones elicit both long-term effects on the neurendocrine status of a recipient animal and short-term effects on behavior. Activation of the VNO neurons is thought to trigger innate or "hardwired" behaviors.[26] Investigation of mouse vomeronasal receptors has begun to dissect out the molecular mechanisms of pheromone signaling from the VNO to the AOB and to relate them to specific social behaviors. There are two independent families of VNO-specific receptors—the V1R family and the V2R family—expressed in spatially segregated populations of neurons. The M1 and M10 groups of MHC Class Ib molecules have now been shown to form complexes with β_2m and the V2R receptor family subset. Mice that are deficient in β_2m and thus cannot express stable pMHC complexes on the surface of cells, can be discriminated from β2m positive littermates by odortype.[27] Also, β_2m knockout mice exhibit deficits in pheromone induced male-male aggressive behavior.[28] Female triple-knockout mice deficient in murine Class Ia molecules H-2K^b, H-2K^d and β_2m exhibit significant deficits

in maternal nurturing behavior, leading to reduced litter survival (personal observation, D. Gould and D. Schust).

TRP2, is a putative ion channel expressed in the VNO and potentially involved in signal transduction pathways triggered by vomeronasal receptor binding. Mice deficient in TRP2 show an absence of VNO sensory activation by pheromones in urine. Pronounced behavioral effects result, with male TRP2-deficient mice failing to display normal aggressive responses to intruder males. TRP2-null males are also apparently unable to gender-discriminate; they display courtship and mounting behavior equally toward both males and females.[29] Lactating TRP2-deficient females also exhibit an unusual lack of aggression against intruders.[30] Current evidence suggests that the VNO became vestigial in the common ancestor of Old World monkeys and apes, and it has been proposed that pheromone signaling may have been replaced by color vision in higher primates and humans.[31] The M1 and M10 MHC Class Ib gene families, associated with β_2m and V2R receptors in the VNO of rodents, are not represented in the human genome.[10]

Different MHC Class Ia and Class Ib genes are expressed in unique subsets of neurons in the central nervous system (CNS), suggesting functional diversity among the genes expressed in this tissue. Neuronal MHC Class I expression corresponds temporally and spatially with well-established areas of activity-dependent development and plasticity in the mouse CNS.[32] These unexpected roles of Class I genes in the CNS, MOB and AOB suggests that MHC Class Ia and Ib gene interactions may originally have evolved as a genetic incompatibility system driving reproductive and rearing behavior, and subsequently coopted for immune recognition. Sexual selection and reproductive fitness are, after all, selective pressures as powerful as the ability to resist infection[33,34]

MHC Class I in Preimplantation Embryos

In humans, fertilization of the oocyte by spermatozoa typically occurs in the distal portion of the fallopian tube, the tubal ampulla. Binding and fusion of sperm and oocyte membranes at fertilization promote two major events: oocyte changes that block polyspermic fertilization and resumption of oocyte meiosis, with release of the second polar body. In vitro, male and female pronuclei form at a median of 8 hours post-insemination and can be reliably identified microscopically approximately 18 hours after fertilization.[35] By 24 hours post-fertilization, pronuclear membranes have disappeared, parental chromosomes have intermingled and the first cellular division occurs. The stages of preimplantation embryo development, which are similar in mouse and human, are depicted in Figure 1. The preimplantation period lasts 4-5 days in mice and 5-7 days in humans.

The initial cell divisions occur in the absence of messenger RNA synthesis, and appear to be driven exclusively by maternal cytoplasmic signals, an occurrence termed the "maternal legacy".[36] These signals are hypothesized to originate within maternal mitochondrial DNA, which does replicate during early embryonic cell divisions. The point at which the paternal genome is activated to undergo transcription is called zygotic gene activation (ZGA).[37] ZGA in embryos is first detected 2-3 days after fertilization when the embryo consists of 4 to 8 cells.[36] In the mouse, ZGA occurs earlier, at the two-cell stage.[38] It is important to note that early cellular divisions occur without a perceptible increase in embryo size and each individual cell within the embryo is totipotent until at least the 8 cell stage. Removal of a single cell or blastomere before this stage can occur without disruption of embryonic development.[39,40] Continued cell divisions occur while the embryo moves proximally within the oviduct toward the intrauterine cavity. By the time this cavity is reached, the developing embryo has completed the 16 cell, solid morula stage and has progressed to the cavitating 32-64 cell, blastocyst stage. Although differential gene expression has occurred earlier, it is not until the blastocyst stage that two types of cells can be easily differentiated microscopically. At the blastocyst stage, the developing embryo has an identifiable, fluid-filled cavity, called the blastocoel, a surrounding layer of trophectoderm that will contribute to formation of the placenta, and an inner cell mass that

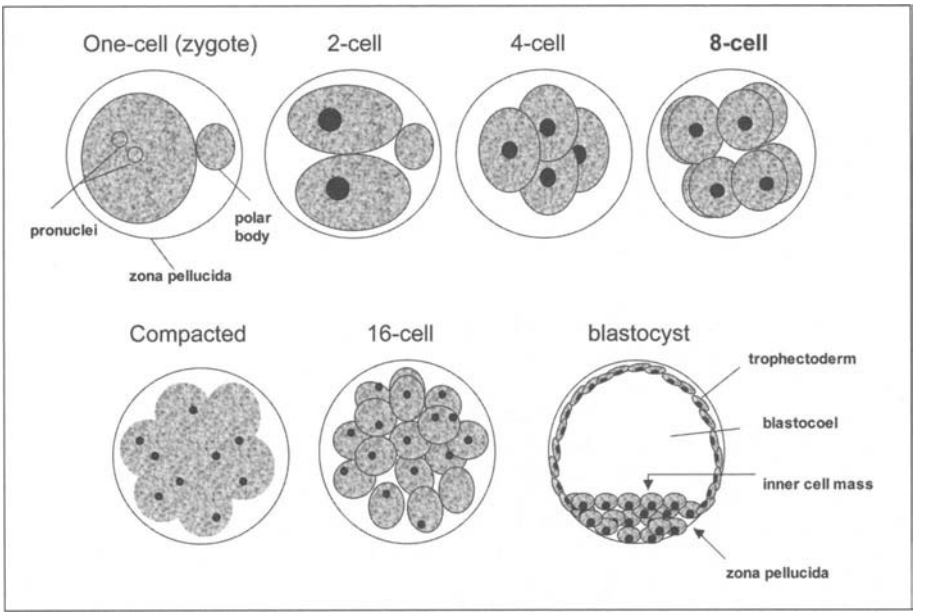

Figure 1. Preimplantation embryo development. Stages of preimplantation embryo development are similar in mouse and human. The preimplantation period lasts 4-5 days in mice and 5-7 days in humans. Early cellular doubling divisions occur without a perceptible increase in embryo size. The zona pellucida surrounds the embryo until implantation.

will form the fetus and some extra-embryonic tissues. Just prior to implantation in the uterus, the blastocyst 'hatches' from the zona pellucida that has surrounded it since the oocyte stage.

Initial studies of MHC Class Ia and Class Ib mRNA and protein expression in mouse preimplantation embryos reported the absence of MHC Class I antigens before mid-gestation.[41,42] As more sensitive techniques became available, expression of mRNA and protein for Class Ia[43-45] and Class Ib[46-49] molecules was detected on all mouse preimplantation stages from oocyte to blastocyst. Class I MHC proteins on mouse embryos are capable of functional interactions with T cell receptors, and cytotoxic T lymphocytes (CTL) can recognize and kill Class I-expressing embryos after removal of the zona pellucida.[50] This suggests that the zona pellucida may play an immunoprotective role during early embryonic development.

Early studies on human preimplantation embryos also reported the absence of MHC gene expression.[51,52] There are, to date, no reports of MHC Class Ia HLA-A, -B, or -C mRNA or protein expression in human preimplantation embryos. Evaluation of mRNA expression for Class Ib genes HLA-E, and -F using RT-PCR in a sample of 108 spare day three human preimplantation embryos from 25 couples indicates that HLA-F is not transcribed while 84% of the embryos were positive for HLA-E mRNA.[53] Three studies of HLA-G mRNA expression in almost 300 embryos from oocyte to blastocyst stage report that 40 to 90% of the embryos were positive for HLA-G mRNA.[53-55] One study using eleven embryos reported no HLA-G message expression,[56] indicating that there may be marked variability in HLA-G mRNA expression in embryos and that sample size may be particularly important. The presence of HLA-G protein in preimplantation embryos has also been reported, and despite varying expression levels, HLA-G mRNA expression intriguingly correlated with a faster cleavage rate of the embryos.[54]

Soluble isoforms of HLA-G protein have been identified in supernatants from human embryos cultured in vitro. Preliminary reports indicate that the presence of soluble HLA-G in tissue culture supernatant may correlate with more successful pregnancy outcome.[57-59] Human embryos fertilized in vitro at the same time exhibit a range of rates of development and, notably, embryos that develop faster exhibit preferential survival. The rate of development is one of the characteristics used in the evaluation of embryos (a component of the embryo quality score) for transfer to recipient mothers during in vitro fertilization procedures.[60] These observations echo the phenotype of the *Ped* (preimplantation embryonic development) gene in mice, which influences the rate of preimplantation embryonic development and subsequent embryonic survival.

Qa-2, The Preimplantation Embryo Development (*Ped*) Gene Product

Qa-2, the mouse *Ped* gene product, is a MHC Class Ib protein with a defined function in regulation of preimplantation embryonic development. Embryos expressing Qa-2 exhibit a faster cleavage rate during preimplantation development. The effect is independent of the maternal environment as Qa-2 positive embryos also develop faster in vitro.[61] Removal of Qa-2 using enzymatic or antisense microinjection techniques slows the rate of development,[62,63] while microinjection of Q7 and/or Q9 DNA encoding the Qa-2 protein increases the embryonic cleavage rate.[64] Beyond the preimplantation stage of development, the *Ped* gene also confers survival advantage to term.[65] Postnatally, Qa-2 positive mice exhibit enhanced birth and weaning weights.[66] Studies are ongoing to determine whether Qa-2-expressing mice have additional survival advantages as adults. Warner and Brenner include a comprehensive overview of the *Ped* gene and the search for its human homolog in their extensive review of the genetic regulation of preimplantation embryo survival.[67]

HLA-G Is the Proposed Human Functional Homolog of Mouse Qa-2

HLA-G and Qa-2, like all mouse and human Class I proteins, are structurally similar and interact with receptors of the acquired and innate immune systems. The receptors of the innate immune system in mice, while exhibiting similar functions to the human NK receptors, derive from different gene families than in the human in a striking example of convergent functional evolution. Analysis of mouse-specific gene clusters expanded after divergence from the mouse-human common ancestor revealed that reproduction and host defense and immunity are major functional themes of the expanded gene clusters in the mouse, indicative of strong evolutionary pressure.[68] Concordant with the roles of MHC Class Ib genes in both immunity and reproduction discussed in this chapter, there is evidence that these are intertwined and fundamental processes subject to selective pressures at the species level.

Orthologous genes have been defined as genes that are related by vertical descent from a common ancestor and encode proteins with the same function in different species.[69] Orthologous relationships of MHC Class I genes have not been found among different mammalian orders where the number of Class I genes is highly variable. Convergent evolution can, however, result in nonorthologous genes encoding proteins with similar functions as is seen in the case of HLA-E in the human and Qa-1 in the mouse[10] and HLA-G in the human and Qa-2 in the mouse.[70] Similarities noted between HLA-G and Qa-2 and summarized in Table 1.[71] suggest that HLA-G is the most likely human functional homolog of the mouse *Ped* gene despite one apparently major difference between the molecules, namely their method of insertion into the cell membrane.

Qa-2 protein is glycosylphosphatidylinositol (GPI)-linked in the outer leaflet of the phospholipid bilayer of the cell membrane. GPI linkage occurs as a post-translational modification of proteins in the endoplasmic reticulum, and the presence of a GPI 'tail' targets proteins to detergent-insoluble membrane domains enriched in glycosphingolipids and cholesterol. The lipid domains or 'rafts' seem to correspond to distinct membrane microdomains with a more ordered liquid phase than the bulk cell membrane. Although they lack a transmembrane

Table 1. Comparison of HLA-G and Qa-2, the* Ped *gene product

Feature	HLA-G	Qa-2
MHC Class Ib molecule, binding nonapeptide	Yes	Yes
Membrane-bound and soluble isoforms	Yes	Yes
Expression by preimplantation embryos	Yes	Yes
Increases preimplantation growth rate	Yes	Yes
Enhances fetal survival	Yes	Yes
Increases birth weight	Unknown	Yes
Increases weaning weight	Unknown	Yes
Short cytoplasmic tail	Yes	Yes
GPI linkage of short tail to membrane	No	Yes
Increased cell proliferation with cross-linking	Unknown	Yes
Acts as a signal transduction molecule	Unknown	Probable

domain, ligation or clustering of GPI-anchored proteins on the cell surface can initiate signal transduction through activation of acylated kinases constitutively present in the cytoplasmic leaflet underlying the rafts.[72] HLA-G is not a GPI-linked molecule, but unlike Class Ia MHC proteins that contain a standard hydrophobic transmembrane domain and a 30-40 amino acid cytoplasmic domain, HLA-G has an abbreviated six amino acid cytoplasmic tail. A diagram comparing Qa-2 and HLA-G is shown in Figure 2.

Functional homology of Qa-2 and HLA-G implies that the short cytoplasmic tail of the HLA-G molecule is equivalent to the GPI-linkage of Qa-2 in permitting HLA-G to localize to lipid rafts, and invoke raft-associated signaling pathways. Alternative use of GPI-anchored

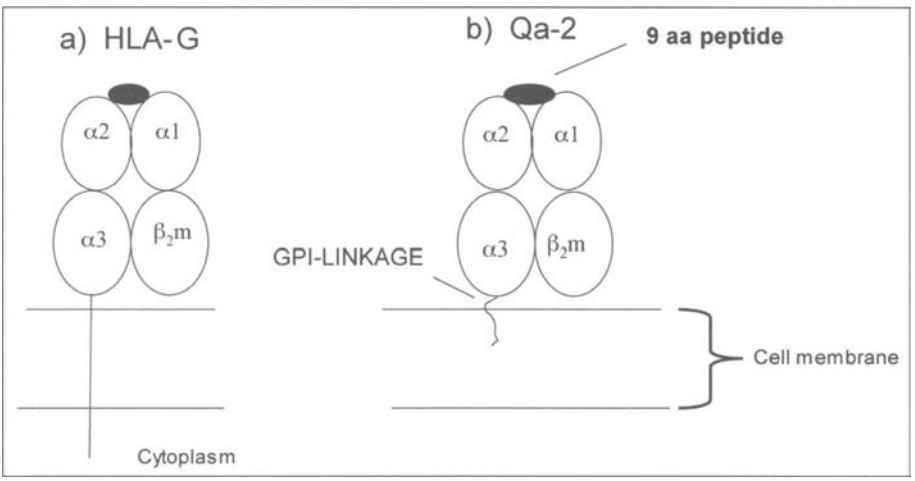

Figure 2. Comparison of the structure of HLA-G and Qa-2. The extracellular domains of the proteins are similar consisting of a trimer complex of three noncovalently bound components: the MHC Class I heavy (alpha) chain containing alpha 1, alpha 2 and alpha 3 domains, the small 9-11 amino acid displayed peptide, and beta$_2$ microglobulin (β_2m). HLA-G has a transmembrane domain and a truncated cytoplasmic domain consisting of 6 amino acids. Qa-2 is post-translationally modified by the addition of a glycosylphosphatidyl inositol (GPI) tail which anchors the protein in the outer leaflet of the cell membrane. Qa-2 does not contain transmembrane or cytoplasmic domains.

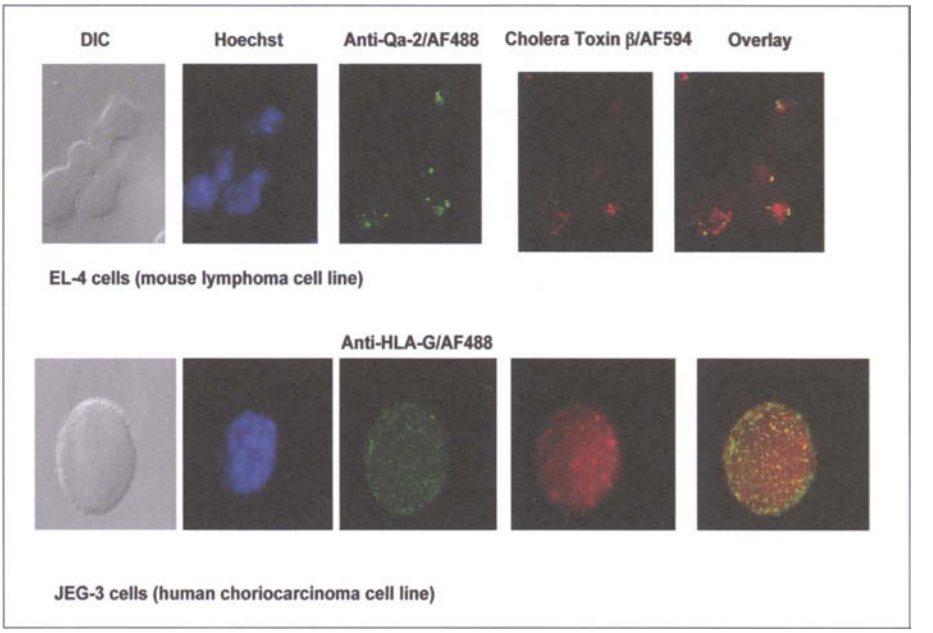

Figure 3. Qa-2 and HLA-G colocalize in lipid rafts. MHC class Ib species are found in lipid rafts in mouse and human. Immunofluorescence colocalization of Qa-2 with lipid rafts in the mouse EL-4 lymphoma cell line (top row) and of HLA-G with lipid rafts the the human choriocarcinoma cell lines JEG-3 (bottom row). Each panel shows in sequence from left to right a DIC image of the cells, nuclear staining of the cells with Hoechst (blue), lipid raft labeling with Cholera Toxin β subunit conjugated with Alexa Fluor 594 (red) and Qa-2 or HLA-G labeling with monoclonal antibody and detected with a secondary antibody conjugated with Alexa Fluor 488 (green). The overlay panels show in yellow the colocalization of Qa-2 (top) and of HLA-G (bottom) with lipid rafts.

versus short cytoplasmic-tailed protein homologs in rodents and man is not without precedent in the literature. It has been reported that in carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), a short cytoplasmic domain isoform in rodent leucocytes has been functionally replaced by a GPI-linked isoform in the human.[73] Preliminary investigation of the association of HLA-G with lipid rafts indicates that the molecule can indeed associate with raft microdomains in the cell membrane.[71] Figure 3 contains immunofluorescence images showing colocalization of Qa-2 and of HLA-G with lipid raft marker GM1 in mouse EL-4 lymphoma cell line and human choriocarcinoma cell lines respectively. Lipid rafts are labeled with Cholera Toxin β subunit conjugated with Alexa Fluor 594 (red). Anti-Qa-2 and anti-HLA-G are labeled with a secondary antibody conjugated with Alexa Fluor 488 (green). The overlay panels show, in yellow, colocalization of Qa-2 and of HLA-G with lipid rafts. Molecules interacting with Qa-2 or HLA-G in mouse or human preimplantation embryos respectively have not been defined, although intensive research is underway to identify Qa-2 interacting molecules in the mouse model. Initially focusing on molecules known to interact with Qa-2 in the immune system, this work does not preclude the existence of novel associations/functions during preimplantation development.

Implantation and MHC Class I in the Trophoblast

Implantation of the embryo in the uterus is a complex process, controlled by both maternal and embryonic signals (reviewed in refs. 74,75). During this process, the embryo becomes completely embedded within the maternal intrauterine endometrial lining, which has

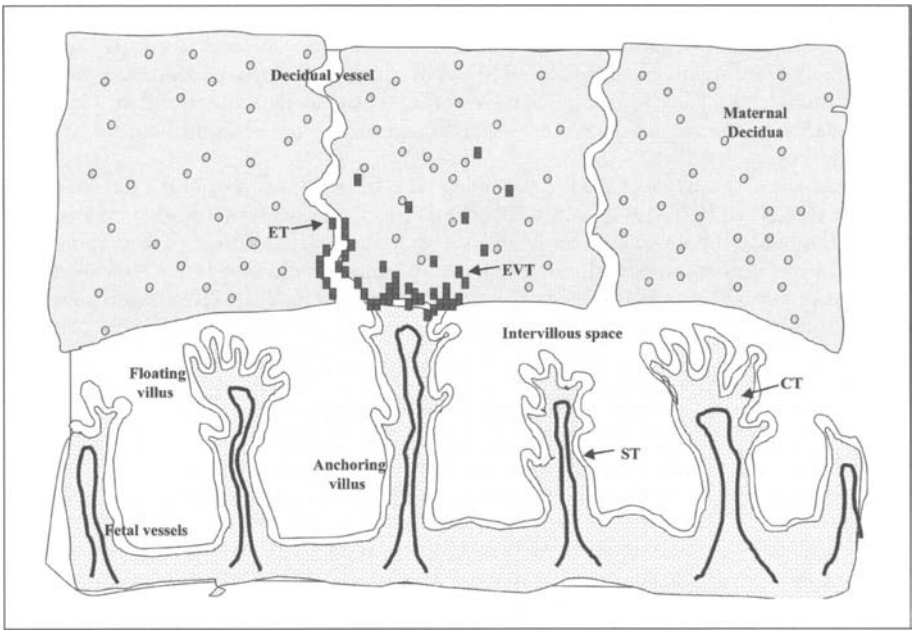

Figure 4. Human placenta microarchitecture. Fetal derivatives in the placenta consist of fetal vessels and placental cotyledons (villae). Villae consist of fetal vesels surrounded by cytotrophoblast cells (CT). Covering the cytotrophoblast cells is a multinucleated cellular layer called the syncytiotrophoblast (ST). Anchoring villae are in direct contact with the maternal uterine lining, called the decidua. The decidua is traversed by maternal vasculature. Blood from these vessels empties into the intervillous space and bathes the placental villae. Cytotrophoblast cells from anchoring villae can change into an invasive phenotype called extravillous cytotrophoblast cells (EVT). EVT invade deeply into the maternal decidua. Some EVT, called endovascular trophoblast cells (ET), embed within the walls of the maternal vasculature.

prepared for implantation by transforming into a metabolically-active, secretory entity called the uterine decidua (Fig. 4). During implantation, trophectoderm cells begin to differentiate into the cellular subtypes that will characterize the mature human placenta. The human hemochorial placenta is comprised of a mass of arborized placental cotyledons (villae), derived from embryonic precursors and bathed in maternal blood. Maternal blood flows into the space between the maternal decidua and the placental villae (the intervillous space) via low resistance, high flow vessels branching from the maternal decidual spiral arteries. The core of each placental cotyledon is traversed by fetal vessels. These vessels are, in turn, surrounded by layers of trophoblast cells. The inner layers of cells are called cytotrophoblast cells (CT). The outer layer of the placental villae is coated by a syncytium of fused, multinucleated cells, called the syncytiotrophoblast (ST) (see Chapter by Guller et al).

Most placental villae are free-floating within the intervillous space. They originate from fetal tissues and are wholly bathed in maternal blood. A subset of anchoring villae completely traverses the intervillous space and attach directly to the maternal decidua. Within these structures, a subpopulation of extravillous cytotrophoblast cells differentiates into an invasive phenotype. Extravillous cytotrophoblast cells differ from their villous counterparts in the expression of cell surface molecules including MHC[76] and proteinase products.[74] They aggressively invade into the maternal decidua, here coming into direct contact with maternal decidual lymphocyte subpopulations. A subpopulation of the extravillous trophoblast cells (EVT) will invade into the maternal vasculature itself, replacing some of the muscular and supporting cells within the wall of the decidual vessels.[77,78] These endovascular trophoblast cells (ET) are felt to

be important in transforming decidual vessels into low resistance, high flow structures, thereby protecting maternal-to-fetal nutrient transfer. The intimate apposition of maternal and fetal tissues and the continuous exposure of fetal tissues to circulating maternal blood are at the core of the immunological paradox first posed by Medawar—how are the embryo and the placental embryo-derived tissues expressing paternal MHC antigens protected from the maternal immune response?

Trophoblast cells are one of the few physiological cellular populations in the human body that lack typical MHC Class Ia (HLA-A and HLA-B)[79] and MHC Class II products.[80,81] Syncytiotrophoblast and villous cytotrophoblast cells are completely devoid of these products, and some investigators promote this as important in maternal tolerance to the fetal allograft. The invasive extravillous cytotrophoblast and endovascular trophoblast cell subsets, however, express a unique subset of MHC Class I products: HLA-C, HLA-G and HLA-E.[82-86] The function of these cell surface molecules remains enigmatic, though many theories have been promulgated. The vast majority of theories concern direct and indirect mechanisms that alter adaptive and/or innate immunity; however, one intriguing series of investigations suggest that the expression by fetus-derived cells of any or all of the trophoblast MHC Class I products could promote essential decidual and vascular invasion.[87] In support of this, alterations in trophoblast expression of HLA-G has been linked to disorders of placental invasion, including preeclampsia.[87,88]

In considering the function of trophoblast Class I species, it may be instructive to discuss important ligands for Class I products that are present on the surface of resident decidual lymphocytes. Like lymphocyte populations at other mucosal sites,[89] decidual lymphocyte subpopulations differ dramatically from those typical of the peripheral immune compartment.[90,91] The human endometrium is normally populated by T cells, macrophages, NK-like cells, and a very limited number of B cells,[92] but during the late luteal phase and in early pregnancy, nearly 70 to 90% of endometrial lymphocytes are believed to be variants of natural killer cells.[93-95] These unusual cells have been variably called decidual granular lymphocytes (DGLs), large granular lymphocytes (LGLs), and decidual natural killer cells.

Natural killer cells are a part of the innate immune system, poised for rapid, antigen-independent recognition of the absence of MHC Class I molecules,[96] the "missing self" hypothesis. The ligands on the surface of NK cells recognize MHC Class I products that can be either inhibitory or activating (reviewed in ref. 97). NK cell receptor categorization is complex and differs among species. In humans, NK receptors fall into two major subcategories: immunoglobulin-like, killer inhibitory receptors (KIRs), and lectin-like heterodimers comprised of CD94/NKG receptor complexes. KIR molecules of both the activating and inhibitory subtypes have been shown to recognize HLA-C locus molecules,[98] while both activating and inhibitory CD94/NKG receptor complexes recognize HLA-E molecules.[97,99] HLA-G can also interact directly with diverse NK receptors, with some outcomes dependent on the activation state of the cell.[100]

Interactions between trophoblast Class I species may allow HLA-G to indirectly control NK cell function in addition to its direct interactions. To this point, HLA-E species have been shown to utilize signal sequence-derived peptides as their essential "antigenic" component allowing stable cell-surface expression of the trimolecular Class I complex.[101-103] Peptides derived from the HLA-G leader sequence are particularly suited to binding within the HLA-E antigen-binding cleft so that generation of the leader peptide during HLA-G synthesis could control cell surface expression and receptor interactions of HLA-E in the placenta. The binding affinity of HLA-E for the inhibitory receptor CD94/NKG2A is usually higher than its binding affinity for the activating receptor CD94/NKG2C. However, the HLA-G leader sequence peptide complexed with HLA-E binds activating receptor CD94/NKG2C with an affinity high enough to trigger an NK cell response (reviewed by Moffett-King[34]). Coexpression of HLA-E and HLA-G in the invasive extravillous and endovascular trophoblast cells therefore suggests that either HLA-G itself interacts with an inhibitory receptor in NK cells, or both

molecules act coordinately with HLA-C expressed in the same cell to regulate interactions with inhibitory and activatory NK receptors. HLA-C products exhibit considerable polymorphism, but have a relatively short half-life at the cell surface,[86] which may limit their efficacy in antigen presentation. The short half-life of HLA-C on the cell surface is also in marked contrast to the unusual stability of HLA-G on the cell surface of the same cell populations.[104] HLA-C has, however, also been reported to be the dominant Class I molecule preventing killing by NK cells. Moreover, it binds to KIR inhibitory molecules with association and dissociation rates that are among the fastest kinetics seen in immune system interactions.[105] The rapid kinetics of HLA-C interactions with KIR may be sufficient to counteract the short half-life on the surface of the cell. KIRs specific for HLA-C are expressed by a higher percentage of uterine NK cells than peripheral-blood NK cells in pregnant women, suggesting that NK-cell specificity is skewed towards HLA-C in the uterus,[34] just as endometrial lymphocytes in early pregnancy are skewed towards NK cells.

CD94/NKG receptors are conserved between rodents and primates, but the KIR receptor families evolved in primates after the separation of rodent and primate lineages. In mice, the equivalent receptors are lectin-like multigenic Ly49 molecules, whereas the single Ly49 gene in humans is nonfunctional. Comparison of KIR receptor evolution in humans and chimpanzees indicates that while NK cells of both species express inhibitory KIR receptors with identical specificity for structural components of HLA-C, the receptors themselves are structurally divergent and not orthologous. The differences between the human and chimpanzee HLA-C-specific KIR are not consistent with neutral evolution and the high sequence similarity in the respective genomes.[106]

Interactions between trophoblast Class I molecules and other decidual lymphocytes also suggest the importance of Class I molecules in innate immunity at the maternal-fetal interface. Many of the lymphocyte subpopulations that are rare in the peripheral circulation but enriched among decidual lymphocytes are important in innate immunity. These include NKT cells, a newly-described lymphocyte subpopulation with characteristics of both NK cells and T cells.[107] In animal models, the presence and quantity of NKT cells at implantation sites is associated with fetally-expressed MHC Class I or Class I like products.[108,109] NKT cells and their ligands have been recently demonstrated in human decidual tissues.[110]

While very few B cells populate the human maternal decidua, T cells are present including those expressing CD8.[90,92,111] The presence on these cells of CD8, a classical ligand for MHC Class I interactions, suggests the possibility of a role for trophoblast Class I products in adaptive immunity at the maternal-fetal interface presumably via classical mechanisms. HLA-G has been shown to interact with T cell-expressed CD8 in vitro,[112,113] and the peptide-binding cleft of HLA-G can bind a diverse set of antigenic peptides.[114] Soluble forms of HLA-G, products of spliced variants of HLA-G mRNA lacking their transmembrane segment,[115-117] have been described in amniotic fluid,[118] in maternal peripheral blood,[119] and in spent embryo culture media.[58,59] Soluble classical Class Ia molecules have been shown to promote immune tolerance[120,121] and Solier et al.[122] have demonstrated that soluble HLA-G molecules specifically promote apoptosis of activated $CD8^+$ T cells, indicating that less traditional interactions of MHC Class Ib molecules may be involved in the trophoblast. Indirect effects of trophoblast Class I products on adaptive immunity may also result from alterations in cytokine secretory profiles by immune cells.[93,123] In vitro demonstration that decidual and peripheral immune cells shift toward the Th2 cytokine secretory phenotype when exposed to HLA-G[124] supports this hypothesis.

Arguing against an important role for trophoblast MHC Class I products in adaptive immune responses are sentinel characteristics of the molecules themselves. HLA-E and HLA-G have limited polymorphism. This homogeneity is in distinct contrast to classical MHC Class Ia products, whose remarkable level of polymorphism promotes the generation of exquisite immune specificity. The relatively short half-life of HLA-C at the cell surface weakens its prospective importance as an antigen-presenting molecule. The expression of HLA-C in extravillous

trophoblast cells, given preferential interactions of HLA-C with NK cell KIR receptors and the preferential homing of NK cells to the endometrium during pregnancy, suggest that this molecule is interacting primarily with components of the older innate immune system rather than the adaptive immune system in the context of early pregnancy.

Conclusions and Future Directions

The paradox of fetal protection from the maternal immune response presents not one but two distinct challenges to our current understanding of transplantation immunobiology. Dissection of the unique coordinate expression and function of HLA-E, HLA-G and HLA-C in selected populations of trophoblast cells appears to be a key prerequisite to understanding the intricate and complex interactions of MHC Class I proteins with components of the innate and adaptive immune systems at the maternal/fetal interface. Coexpression of this unique combination of MHC molecules suggests a critical role for MHC Class I molecules in this most fundamental of biological processes. The apparent absence of any MHC Class I expression in other populations of trophoblast cells in the presence of bountiful NK cell populations presents a second type of fetal/maternal interface that challenges our current understanding of the paradigmatic immune response. Unraveling the control and regulatory mechanisms at work in the trophoblast contains the promise of useful application in inhibition or amelioration of context-specific autoimmune conditions. The spatial and temporal difficulties inherent in studying implantation and placental development in early pregnancy in humans, the unique attributes of human placentation, and the limitations of animal models contribute significantly to the challenges. Development of nonhuman primate models and reagents, an expensive and technically difficult undertaking in its own right, may help to bridge the chasms in our understanding, and progress is being made in this regard.[125]

All of the problems involved in studying MHC expression and interactions in the trophoblast are equally valid in asking similar questions about the preimplantation embryo, with the added complication of the absence of federal funding for such studies in the United States. Research is underway to dissect the mechanisms of Qa-2 regulation of preimplantation embryonic development in the mouse, which may shed some light on the role of HLA-G in human embryos. HLA-G transgenic mice may be useful in some studies, and efforts to develop resources using nonhuman primates are under way.[126] Elucidating the role of MHC in human preimplantation embryos is important for understanding the regulation of very early development in addition to understanding the mechanisms of immunoprotection of the preimplantation embryo.

Given the strong selective pressures operating on both the immune and reproductive systems over time, deciphering the role or roles of MHC Class I genes and proteins during preimplantation development and early pregnancy may also offer glimpses of the mysterious evolution of the MHC. An ancestral role for MHC in "self-aware" mate selection and reproductive behavior predating the development of MHC function in the immune system remains a tantalizing possibility. The importance of the unique MHC Class I interactions with the innate immune system in the trophoblast suggests an ancient and robust association that challenges us to expand the standard model of the role of MHC Class I in defense against pathogens and in surgical transplantation. Rather than trying to accommodate models of MHC Class I interactions in the preimplantation embryo and the trophoblast in the light of lessons learned from surgical transplants, the real challenge is to understand the responses evolved in the context of the only natural vertebrate "transplantation" model, the fetal-maternal interface.

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CHAPTER 14

Actions of Seminal Plasma Cytokines in Priming Female Reproductive Tract Receptivity for Embryo Implantation

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Abstract

Embryo implantation is critically dependent on a supportive uterine environment. Uterine receptivity is the culmination of a cellular and molecular transformation mediated locally by paracrine signals under the governance of ovarian steroid hormones, with cells and cytokines of the immune system playing integral roles in this process. Semen is now recognised as contributing to endometrial preparation for embryo implantation, through the agency of specific factors in the seminal plasma fraction of the ejaculate. Transforming growth factor-β (TGFβ) and other immunoactive moieties derived from male accessory glands interact with epithelial cells in female reproductive tissues to induce pro-inflammatory cytokine expression and initiate an inflammatory cascade. The consequences are recruitment and activation of macrophages, granulocytes and dendritic cells which have immune-regulatory and tissue remodelling roles. The cytokines elicited by seminal activation also exert embryotrophic effects and contribute to optimal preimplantation embryo development. This review summarises our current understanding of the molecular and cellular basis of interactions between seminal plasma and the female reproductive tract, and explores the potential mechanisms through which seminal plasma influences the establishment of pregnancy.

Introduction

Exposure to semen elicits striking changes in cytokine expression and in resident leukocyte populations in female reproductive tract tissues. A dramatic infiltration of activated inflammatory cells including macrophages, dendritic cells and granulocytes is evident after seminal contact in the cervix and uterus of all species so far studied. The molecular and cellular basis of this post-mating inflammatory response has been explored most thoroughly in mice.[1,2,3] The response is initiated when seminal plasma moieties interact with estrogen-primed uterine epithelial cells to induce a surge in synthesis of pro-inflammatory cytokines including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6 and an array of chemokines including monocyte chemotactic protein (MCP)-1, KC, macrophage inflammatory protein (MIP)-1α, MIP-1β and RANTES.[2,4] The response is transient, with resolution of inflammation by the time of embryo implantation on day 4 of pregnancy in the mouse. Similar effects are seen in pigs,[5] where instillation of seminal plasma into the uterine lumen at estrus induces expression of GM-CSF, IL-6 and MCP-1, which recruit macrophages and dendritic cells into the endometrial stromal tissue.[6]

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Cellular changes comparable to those seen in the mouse and pig appear to take place in the human cervix. Intercourse is known to elicit neutrophil recruitment into the superficial epithelium of the cervical tissues,[7] but changes in the deeper cervical stromal tissues have been more difficult to study. In a recent study examining the local effects of natural insemination in peri-ovulatory women, we have shown that intercourse induces an inflammatory reaction across the full thickness of the cervical epithelium and subjacent stromal tissues with a striking infiltration of macrophages, dendritic cells, and lymphocytes in both compartments.[8] Leukocyte influx requires contact between seminal fluid and the female tract tissues since no inflammatory response was seen following condom-protected intercourse. Regulation of the cervical leukocytic infiltrate occurs by activation of pro-inflammatory cytokines GM-CSF, IL-6 and IL-8.[9] In vitro studies suggest that the effects of seminal plasma may extend to the uterus in women.[10,11]

In this chapter we will review recent advances in our knowledge of the molecular regulation of this response, including the identity and interaction between active constituents in seminal plasma, and examine the potential physiological consequences in terms of female reproductive function and pregnancy success. The review will largely focus on events in mice, but where relevant information is available, human and other species will be discussed.

Semen Exposure and Pregnancy Outcome

A case for semen exposure contributing to optimal pregnancy outcome can be made based on data from several mammalian species. While the practise of artificial insemination shows that seminal plasma is not mandatory for initiation of pregnancy, there is evidence that the success and quality of the pregnancy, particularly as measured by growth trajectory of the fetus, are compromised if females are not exposed to seminal plasma. Experiments in which the seminal vesicle, prostate or coagulating glands are surgically removed from mice, rats and hamsters prior to mating each show that seminal vesicle fluid is the most vital nonsperm component of the ejaculate.[12-15] In mice, embryo transfer protocols generally employ recipients exposed to seminal plasma by mating to vasectomised males, but fetal loss and abnormality is considerably greater when pseudopregnancy is achieved without exposure to male fluids.[16] When recipient females are mated with seminal vesicle deficient males, transferred embryos yield fetuses with altered growth trajectories and this is associated with changes in placental development.[17] In rats, implantation rates and fetal growth are similarly impaired unless females are inseminated prior to embryo transfer.[18] In pigs, artificial insemination with diluted semen reduces litter sizes but mating with a vasectomised male or administration of heat-killed semen restores litter size and improves farrowing rate.[19,20]

Clinical studies in humans have shown that live birth rates in couples undergoing IVF treatments are significantly improved when women are exposed to semen at the time of embryo transfer.[21,22] Furthermore, treatment of women suffering from recurrent spontaneous abortion with seminal plasma pessaries has been reported to improve pregnancy success.[23] In preeclampsia, there is a cumulative benefit of chronic exposure to semen, with limited sexual experience or use of barrier methods of contraception being linked with increased risk,[24,25] and evidence from women where a change in male partner has occurred suggesting that the effect is partner-specific.[26] Markedly increased rates of preeclampsia are also evident in pregnancies initiated by donor oocytes or semen,[27] when prior exposure to sperm or conceptus antigens has not occurred.

Active Factors in Semen

Experiments in mice from which accessory glands were surgically removed showed that the active inflammation-inducing moieties in semen are derived from the seminal vesicle, the principal constituent of seminal plasma in the mouse.[2] Using protein chromatographic techniques and neutralising antibodies, TGFβ was identified as the key component for induction of uterine epithelial GM-CSF synthesis following mating in mice.[28] The TGFβ content of seminal

vesicle fluid is 70 ng/ml;[29] approximately five-fold the content of serum, and similar to that of colostrum which is the most potent biological source of TGFβ known. Seminal vesicle TGFβ synthesis is testosterone dependent, with a severe reduction evident after castration, and partial recovery after administration of exogenous steroid hormone.[29]

TGFβ is also identified as the principle active moiety in human semen in experiments using primary and transformed human cervical keratinocyte cultures.[10,11,30] This cytokine was initially identified in the plasma fraction of human semen when it was recognised to confer inhibitory bioactivity in prostatic carcinoma cell lines.[31] Unlike TGFβ in serum, which is present exclusively in the latent form complexed with β2-microglobulin, approximately 25% of TGFβ in human and rodent seminal plasma exists in the mature, active form. Subsequently it was shown using isoform-specific immunoassays that TGFβ in human semen is principally of the TGFβ1 isoform, with a lower content (5-10%) of TGFβ2.[32,33] The content of TGFβ3 approximates that of TGFβ1 yielding a final concentration of approximately 300 ng/ml total TGFβ (Sharkey and Robertson, unpublished data). Responsiveness of both murine uterine epithelial cells and human cervical epithelial cells to TGFβ is maximal at ovulation.[10] Whether this reflects a differential expression in TGFβ receptors or other components of the docking or signal–transducing repertoire of molecules remains to be elucidated.

Other inflammation-inducing moieties present in semen are likely to synergise with TGFβ in targeting female tract cells, and may act differentially between species and even between individuals in a population. ϖ Prostaglandin E (PGE) is abundant in human semen as the 19-hydroxy form, but is undetectable in rodent and porcine seminal plasma. In vitro experiments with cultured human cervical explants show that 19-hydroxy PGE promotes expression of chemotactic IL-8 and inhibits expression of the anti-inflammatory molecule secretory leukocyte protease inhibitor (SLPI).[34] Another abundant seminal plasma cytokine is IL-8, which synergises with TGFβ to induce IL-1β, IL-6 and LIF from endometrial epithelial cells.[11]

Bacterial lipopolysaccharide similarly acts to induce cytokine synthesis in murine and human uterine and cervical epithelial cells, presumably through binding to Toll-like receptors TLR2 and TLR4. Of emerging interest is the impact of different 'probiotic' versus pathogenic bacterial species in the male and female tract flora.[35] Through differential binding to TLRs and other pattern recognition receptors on the surface of reproductive tract epithelial cells, the relative abundance of different bacterial species would further influence the character of the cytokine response.[36] Finally, we find that the type-1 cytokine interferon (IFN)-γ acts as a potent inhibitor of TGFβ signalling both in human and mouse epithelial cells.[37] Together this provides an emerging picture of multiple active seminal constituents acting in concert to elicit expression of several cytokines in the female tract, and implies likely variation in the strength and pattern of response elicited by individual seminal fluids within a male population.

Consequences of the Post-Mating Inflammatory Response

The inflammatory response accompanying insemination impacts on several reproductive processes by virtue of the wide variety of potential actions of the leukocytes recruited into the endometrial and cervical tissues. Four categories of effector function are postulated; (1) clearance of superfluous sperm and microorganisms introduced into the uterus at mating; (2) activation of female immune responses specific to paternal transplantation proteins and other antigens present in semen; (3) tissue remodelling associated with preparation of endometrial receptivity; and (4) activation of expression of cytokines and growth factors implicated in preimplantation embryo development (Fig. 1).

The distribution of seminal material within the female tract after coitus would constrain the tissues infiltrated by inflammatory cells and thus the range of downstream effects in a species-specific manner. In rodents and pigs the ejaculate fills the uterine lumen and clearly can directly access the implantation site, but in humans an impact on the uterine environment is more difficult to envisage, with semen deposition occuring at the external os of the cervical canal. Of relevance to this is the observation in humans that seminal plasma constituents including TGFβ are bound to the postacrosomal region of the sperm head and thus presumably

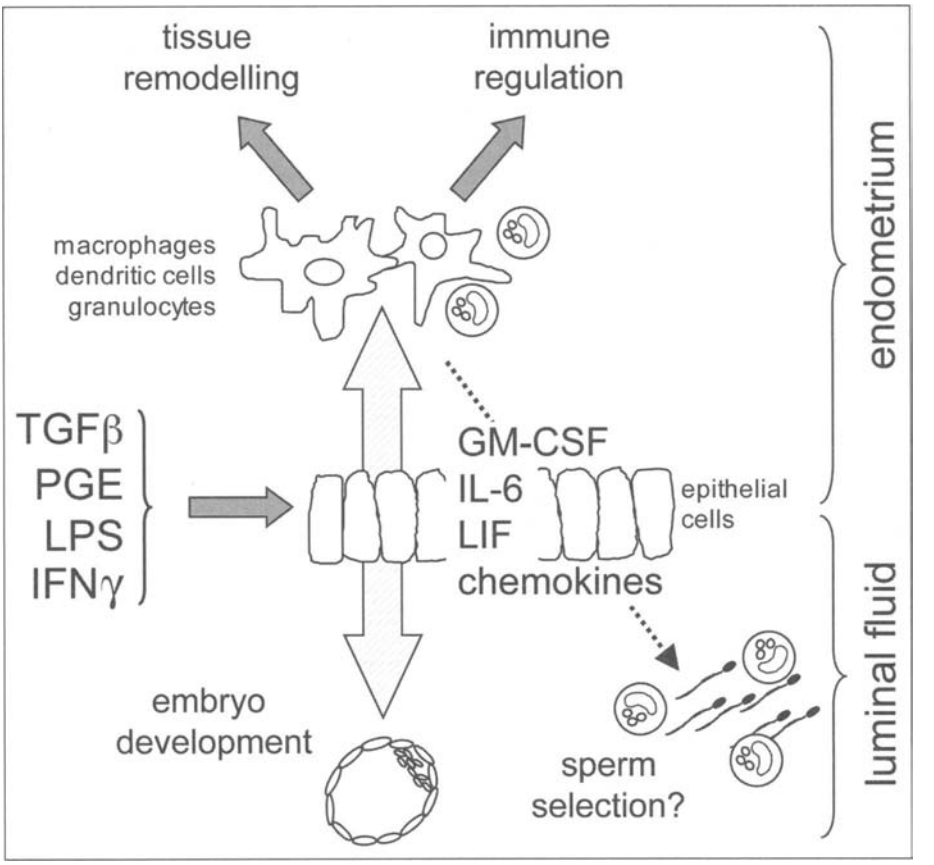

Figure 1. Schematic illustrating potential roles for the post-mating inflammatory cascade in promoting uterine receptivity and embryo implantation. Seminal plasma cytokines including TGFβ, 19-OH PGE and IFNγ, as well as bacterial LPS, synergise to activate expression of pro-inflammatory cytokines in uterine and cervical epithelial cells after mating. Cytokines including GM-CSF, IL-6, LIF and several chemokines such as MCP-1, KC and RANTES act to recruit and activate macrophages, dendritic cells and neutrophils into the endometrial stroma. Infiltrating macrophages and dendritic cells have roles in processing and presentation of seminal antigens to activate maternal immune tolerance. Macrophages further act to secrete growth factors, MMPs and other enzymes and angiogenic molecules to promote tissue remodelling and vasculogenesis. Neutrophils passing into the luminal space participate in clearance of debris and micro-organisms introduced at mating, but may also contribute to sperm selection through phagocytosis of redundant sperm. Cytokines secreted into the luminal fluid target the developing embryo.

are carried together with sperm into the higher tract.[38] Furthermore delivery of seminal plasma as well as sperm from the cervix into the uterus and Fallopian tubes is served by a process of rapid and sustained uterine peristaltic contractions which transport macromolecular material to the tube ipsilateral to the dominant follicle independently of any motile or chemotactic properties of sperm.[39]

Sperm Selection and Clearance of Seminal Debris

One obvious role for the abundant populations of neutrophils that emigrate between epithelial cells into the uterine lumen is phagocytosis and clearance of micro-organisms and seminal debris remaining in the tract after intromission. The higher reproductive tract is normally

sterile, with insemination providing the opportunity for access by commensal micro-organisms originating from male and female tissues including sexually transmitted pathogens. In mice, bacteria are prevalent in the uterus after insemination but sterility is recovered within 24 h, even in GM-CSF null mutant mice where impaired macrophage function predisposes to uterine infection in virgin animals.[40] The physiological significance of seminal plasma in uterine clearance is illustrated in livestock species where rapid resolution of the uterine inflammatory response is linked with pregnancy success.[41,42]

Phagocytic activity in the cervical or uterine lumen targets spermatozoa as well as bacteria. Sperm selection is an interesting potential function of female leukocytes recruited across the luminal surface of the tract; through differential resistance to phagocytosis individual sperm appear to be selected for fertilisation competence and on the basis of other morphological or antigenic parameters,[43,44] such as haplotype in the MHC-linked t-complex.[45] Whether female immune cells can actively select and inactivate sperm within a single ejaculate or distinguish between sperm of different mates in polyandrous species requires investigation. While antibody and complement-mediated opsonisation would provide a potential mechanism for selection, the molecular basis of discrimination and the identity of any target structures remain to be characterised.

Priming the Maternal Immune System to Paternal Antigens

Macrophages and dendritic cells comprise the major populations of cells recruited into the endometrial stromal tissue after exposure to semen. Both have professional antigen processing and presenting ability and are implicated in initiating active immune responses to paternal MHC and other antigens in semen. The consequences of this would be important for future female tract immune responses to semen, as well as for pregnancy, since the conceptus shares paternal antigens with those in semen. Early in the post-mating inflammatory cascade, these cells accumulate subjacent to the surface epithelium in the endometrial stroma,[2,3] then engulf and process paternal ejaculate antigens, before trafficking to para-aortic lymph nodes (PALN) draining the uterus, the mesenteric lymph nodes and spleen.[16,46]

These events result in activation and proliferation of lymphocytes in draining lymph nodes. The PALN of mice enlarge after allogeneic insemination,[47] as T-lymphocyte proliferation commences and expression of cytokines and activation antigens becomes evident.[48] Matings with vasectomised males indicate that lymphocyte activation occurs independently of sperm but in contrast, males from which seminal vesicle glands have been surgically removed fail to stimulate PALN cell proliferation or cytokine synthesis.[48] Amongst the responding cells are T-lymphocytes reactive with paternal MHC antigens, which are aggressively immunostimulatory in graft–versus-host assays for the first two days after insemination and then show evidence of suppressive regulation.[49] Similarly, paternal specific alloreactivity is strongly suppressed in para-aortic lymph node cells recovered on day 3 of pregnancy from rats.[50] Similar kinetics are evident in the time course of expansion of TGFβ-producing suppressor cells identified within the para-aortic lymph node from the time of implantation,[51] and in the appearance of T regulatory cell populations, which have recently been shown to increase in the blood, lymph nodes and spleen of mice within three days after mating.[52]

Induction of Maternal Immune Tolerance for Implantation

Activation of maternal lymphocytes after mating raises the possibility that exposure to semen can impact on the phenotypes or abundance of lymphocyte subsets regulating implantation and placental morphogenesis. In rodents, specific populations of lymphocytes appear within the decidua to promote placental growth and development during the first days after implantation. These lymphocytes include α/β and γ/δ T-cells,[53,54] NKT cells[55] and NK cells.[56] Based on the kinetics of their induction and the similarity in phenotypes between decidual and PALN lymhocytes, we have hypothesised that the appearance of these cells in the implantation site might be causally linked with the female immune response to ejaculate antigens.[48,57] The

possibility that lymphocytes activated and induced to proliferate at insemination might be selectively recruited into uterine implantation sites after recirculation via the blood has been evaluated by passive transfer experiments in pregnant mice. Lymphocytes recovered from the PALN after insemination and radiolabelled prior to passive transfer into pregnant recipients can indeed be shown to home to implantation sites in the uterus.[48]

The lymphocytes present in the implantation site are largely antigen nonspecific T-regulatory cells and NK cells. Uterine NK cell precursor cells are known to originate in tissues other than the uterus, with the spleen identified as the richest source and a lesser contribution from peripheral lymph nodes,[58] so it is unlikely that PALN-derived cells add substantially to this lineage. In contrast, it is possible that NKT cells activated in PALN at insemination home to the uterus and contribute to the dramatically (40-fold) expanded NKT population evident in the implantation site by day 6 of pregnancy.[55] Uterine NKT cells are T-cell receptor $V\alpha14^+$ but express a novel Vβ repertoire reactive with a class I/Ib molecule other than CD1 expressed by placental cells.[59] If semen were to provide or induce in female tissues the yet to be identified class I/Ib molecule, this could provide a mechanism for NKT activation in early pregnancy. This possibility is supported by the presence of MHC class Ia and Ib molecules in semen[60] and of α-galactosylceramide and other glycolipids in sperm.[61]

The smaller contingents of antigen-specific lymphocytes present in the implantation site might reasonably recognise paternal antigens present in semen and shared by the conceptus. Semen contains abundant major and minor histocompatability and other antigens,[62] as well as somatic cells such as leukocytes and desquamated genital tract epithelial cells,[63] and soluble HLA.[64]

The quality of any lymphocyte response raised to seminal antigens would need to be consistent with maternal tolerance of the conceptus at implantation. Seminal plasma contains several powerful immuno-regulatory molecules that can dampen potentially destructive Type-1 (cell-mediated) immune responses and drive immune outcomes of the quality required for functional immune tolerance. Both the PGE and TGFβ present in semen have well described immune-deviating properties in other tissues.[65,66]

Consistent with this, changes in T-lymphocyte status in draining lymph nodes and peripherally in the female after insemination are accompanied by evidence of a transient state of hypo-responsiveness in paternal alloantigen reactive lymphocytes. That semen can induce functional immune tolerance to male antigens was first suggested by experiments showing that mated mice are unable to reject syngenic skin grafts of paternal origin.[67] Subsequently it was demonstrated that protection is similarly conferred to major histocompatibility antigens,[47] but only when sperm is delivered in the context of seminal plasma. Washed sperm, but not whole semen, was shown to elicit transplantation immunity to paternal skin graft challenge, despite both immunisation events leading to lymph node hypertrophy. Likewise, immunisation with washed sperm but not natural insemination primed mice for generation of cytotoxic T-lymphocytes against H-Y antigen.[68] The potential beneficial effect of this immune response for pregnancy outcome has been identified in experiments showing that uterine 'priming' with semen can promote implantation and fetal growth in subsequent pregnancies, in a partner-specific manner.[47,69] Consistent with an immunological mechanism, removal of lymph nodes draining the uterus after exposure to semen revoked the effect and led to a decrease in litter size and fetal and placental weight.[70,71]

Contribution to Tissue Remodelling

In evaluating the impact of the post-mating inflammatory cascade it is important to recognise that leukocytes exert effects in their local milieu other than through activating immune responses. Macrophages and granulocytes secrete an array of potent enzymes and signalling molecules that can elicit proliferation, differentiation or other functional changes in the status of adjacent nonhemopoietic cells (see Chapter 6). Through influencing the structure of the extracellular matrix and the behaviour of endothelial cells, epithelial cells and fibroblasts comprising

the endometrium, tissue remodelling roles which assist in the preparation for pregnancy can be envisaged.

Foremost amongst macrophage- and granulocyte-derived enzymes are the matrix metalloproteinases (MMPs), a family of zinc-containing endo-proteinases that share structural domains but differ in substrate specificity and regulation of synthesis. Macrophage production and secretion of large quantities of MMPs is regulated by local cytokine environment, with induction after exposure to factors including GM-CSF, tumor necrosis factor alpha (TNF)α and IL-1. The catalytic activity of these enzymes is pivotal for cyclic endometrial breakdown and regrowth, and for the remodelling underlying embryo implantation and decidualisation.[72] Precise spatial and temporal patterns of expression of the MMP family and its regulatory component, the tissue inhibitors of metalloproteinases (TIMPs), are characteristic of the pre and peri-implantation period in rodents.[73] The significance of leukocytes recruited in response to seminal factors in regulating MMPs during early pregnancy has not been evaluated but consistent with such a role is observations in rats that expression of MMP-7 (matrilysin) is highest on the first day after mating,[74] with MMP-2 also induced prior to embryo implantation.[75] In golden hamsters, induction of pregnancy in the absence of male accessory gland fluids is associated with reduced expression of MMP-2 in the implantation site.[76]

Inflammatory leukocyte regulation of endothelial cells in the angiogenic response provides another potential avenue for seminal factor effects on implantation. Vasodilation and oedema are associated with the inflammatory response to semen in mice and several other species.[6,77] Vascular endothelial growth factor (VEGF) as well as other key angiogenic factors IL-1, TNFα and basic fibroblast growth factor (bFGF) are identified as products of activated macrophages and clearly are candidate mediators of the endothelial changes induced by semen.[3,78] That VEGF mRNA expression in the implantation site is diminished when pregnancy is initiated by accessory-gland deficient males in golden hamsters suggests that the consequences of semen-induced angiogenic changes perpetuate beyond the acute inflammatory period.[76]

A further target for the actions of macrophage-secreted products in early pregnancy are the luminal epithelial cells involved in embryo attachment during the initial phases of embryo implantation. The 'window of implantation' or opportunity for embryo adhesion is defined by specific changes in the expression of epithelial integrins and mucins, allowing close apposition between the blastocyst and the luminal surface, and finally adhesion of the two cell surfaces.[79] While ovarian steroid hormones clearly have an overarching role in regulating these changes, macrophages are closely juxtaposed with processes indigitating between epithelial cells in the endometrium, and this spatial association affords a potential role in influencing integrin expression at the paracrine level. That leukocytes may directly regulate the adhesive properties of epithelial cells has been demonstrated with human uterine epithelial cells in vitro, using membrane spheroids from the choriocarcinoma cell line BeWo.[80] The ability of macrophages to alter transport properties and maintenance of epithelial barrier integrity[81] might further contribute to implantation through facilitating trophoblast breaching of the epithelial surface.

Activation of Embryotrophic Cytokines

The cytokines induced after exposure to semen target not only maternal leukocytes in the endometrial stromal tissue, but also are secreted into the luminal space to potentially interact with the developing embryo as it traverses the oviduct and uterus prior to implantation. Several cytokines activated by semen are amongst those attributed with regulating proliferation, viability and differentiation of blastomeres in embryos.[82,83] GM-CSF, a major component of the post-mating cytokine response in mice,[28,84] is identified as essential for normal blastocyst development and subsequent viability. Expression during early pregnancy also occurs in the uterus and oviduct of women[85] and other mammalian species.[86,87] GM-CSF targets the preimplantation embryo to promote blastocyst formation, increasing the number of viable blastomeres through inhibiting apoptosis and facilitating glucose uptake.[88] Human embryos cultured in GM-CSF are twice as likely to reach the blastocyst stage of development, blastulate earlier and

have increased cell numbers both in the inner cell mass and trophectoderm.[89] Other cytokines targeting the developing blastocyst including IL-6 and LIF are similarly induced after exposure to semen[11,90] (SAR unpublished data).

Summary and Conclusions

A significant body of evidence now exists to link exposure to semen with pregnancy success in human, rodents and several additional mammalian species, and the studies reviewed herein are beginning to provide explanations for the underlying molecular and cellular mechanisms. Seminal plasma can thus no longer be considered simply a sperm transport medium, but instead must be recognised as a means for communication between the male and female reproductive tissues. This function of seminal plasma presumably has its evolutionary origins in benefiting the likelihood of a pregnancy after insemination by a given male. From the female perspective the opportunity for activation of immune events prior to implantation may facilitate sperm selection and discrimination between competent and suboptimal embryos.

To date, research in this field has focussed largely on rodent and livestock species, and for obvious reasons the significance of seminal factors in humans have been more difficult to explore. While direct extrapolation from the rodent to the human may not be justified, clearly these findings have implications for the association between semen exposure and the incidence of pathologies of human pregnancy. We speculate that the aberrant Type 1 immunity associated with 'shallow' placentation in preeclampsia and recurrent miscarriage[26,91] can be initiated by insufficient or inappropriate immune responses to seminal antigens following intercourse, perhaps linked with seminal plasma cytokine deficiency or female incapacity to respond to seminal signals. There are additional implications for assisted reproductive technologies, where pregnancies are routinely initiated in the absence of natural intercourse. A better understanding of the physiological significance of semen in human implantation requires further detailed exploration of the cellular and molecular events within the female reproductive tract at insemination, and may eventually yield novel therapies for infertility and pathologies of pregnancy.

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CHAPTER 15

B7 Family Molecules in the Placenta

Margaret G. Petroff

Abstract

The mechanisms of acceptance of the fetus by the maternal immune system are mediated in part by immunomodulatory proteins expressed by placental cells. The recent discovery of novel members of the B7 family of immunomodulators has prompted much excitement among the scientific community because of their role in immunological tolerance. Many members of the B7 family are now known to be expressed in the placenta, suggesting that they contribute to the modification of the surrounding immunological milieu such that the fetal allograft is tolerated. In this chapter, we will summarize recent findings regarding the expression and possible functions of B7 proteins in the placenta.

Introduction

Peaceful coexistence between the mother and fetus during gestation involves a complex molecular dialogue that ensures proper adaptation of the maternal immune system to the genetically dissimilar fetus. This dialogue takes place between placental cells and maternal blood and decidual cells, and results in modification of maternal lymphocyte function. Some changes can be attributed to alterations in the hormonal environment that occur in pregnancy. However, it has become increasingly apparent that trophoblast cells themselves interact with maternal lymphocytes, either through direct cell-cell contact, or indirectly through secretion of soluble macromolecules.

Immunological tolerance is defined as the inability of immune cells to respond to an antigen. The loss of immune tolerance toward self-antigens results in activation of immune cells that target and destroy self-tissues. The best-understood site of acquisition of tolerance is within the primary lymphoid organs, particularly the neonatal thymus. However, acquisition of tolerance in the thymus fails to explain the lack of immunological aversion to tissue-specific antigens. With this in mind, researchers have increased their attention toward understanding the mechanisms of tolerance to peripheral tissues and antigens, and further, to the breakdowns in these mechanisms that occur in autoimmune disease. The placenta, with its expression of paternal genes within the immunologically foreign maternal environment, is a prime example of an organ requiring the generation of effective T cell tolerance. Indeed, studies using transgenic mice have shown that populations of T cells that specifically recognize fetal antigens are deleted or tolerized.[1] It is widely accepted that maternal tolerance to the placenta is achieved in successful pregnancy, both passively, as exemplified by the lack of polymorphic human leukocyte antigen (HLA) expression in the villous placenta, and actively, through molecular suppression of lymphocytes.[2]

B7 family molecules are type I transmembrane proteins belonging to the immunoglobulin (Ig) superfamily. B7 proteins mediate cell-to-cell interactions through their CD28 family receptors, which are expressed primarily by T lymphocytes. There are currently seven known members of the B7 family and five members of the CD28 family; depending on the ligand-receptor combination, these interactions can have either stimulatory or inhibitory

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Table 1. Summary of B7 family expression in the placenta

Ligand	Alternative Names	Effects on T Cells	Stimulatory Receptor	Inhibitory Receptor	Placental Expression	Refs.
B7-1	CD80	Positive or negative	CD28	CTLA-4	None	8
B7-2	CD86	Positive or negative	CD28	CTLA-4	RNA, protein	8
B7-H2	B7h, B7RP1, hGL50	Positive	ICOS	ND	RNA, protein	Petroff et al, unpublished
B7-H1	PD-L1	Positive or negative	ND	PD-1	RNA, protein	8
B7-DC	PD-L2	Positive or negative	ND	PD-1	RNA, protein	Petroff et al, unpublished; 23
B7-H3		Positive or negative	ND	ND	RNA	60, 62
B7-H4	B7x, B7-S1	Negative	ND	BTLA	RNA	65, 67; Petroff et al, unpublished

ND: not determined

effects on T cells (Table 1). B7-1 and B7-2 were the first two B7 proteins to be discovered; these molecules provide the critical costimulatory signals that allow initial activation of T cells when foreign peptide is presented in the context of major histocompatibility complex (MHC) antigens. These proteins are primarily expressed on professional antigen presenting cells, such as dendritic cells and macrophages. On the other hand, recent research has identified additional pathways involving novel B7 family molecules, revealing their critical role in modulating effector functions of T cells. These molecules, which are more widely expressed, mediate immune responses in peripheral organs, ensuring optimal response to foreign antigens while maintaining tolerance to self-antigens.

Recently, it has been found that nearly all of the B7 family members known to date are expressed within the placenta (Table 1), suggesting that the role of these proteins in organ-specific immunity is paralleled in the placenta. In this chapter, the current state of knowledge of each of the B7 family molecules will be discussed in the context of a proposed role in modulating the maternal immune response to the fetal semi-allograft.

B7-1 and B7-2

The two-signal model of T cell activation has become one of the central tenets of immunology, and was developed when it was recognized that antigen alone is insufficient to cause lymphocyte activation.[3] This theory constituted a key advancement in our understanding of the ability of the immune system to distinguish between self and nonself antigens, and its molecular basis is now well understood. Signal one in T cell activation is delivered to the T cell receptor by a peptide antigen that is presented in context of an MHC molecule by an antigen presenting cell. Alone, however, this signal is insufficient activate naïve lymphocytes. A second signal, provided by costimulatory molecules expressed by professional antigen presenting cells, allows cell cycle progression and clonal expansion. The most effective and probably most important second signal is delivered by ligation of the CD28 molecule, which is constitutively expressed on the surface of all T cells, by either B7-1 (CD80) or B7-2 (CD86).

CTLA-4 is a second receptor for B7-1 and B7-2, but unlike CD28, it is a negative regulator of the immune response. CTLA-4 is rapidly upregulated on T cells after ligation of CD28, and binds to the B7s with a significantly higher affinity.[4] This inhibitory signal is now understood

to be a component of a negative feedback mechanism to systematically down-modulate an active immune response.[5]

Several aspects of the B7/CD28/CTLA-4 signaling system have important implications for immune tolerance to self antigen. In the absence of the second signal during T cell activation, T cells either fail to respond, become anergic and unable to respond, or undergo programmed cell death.[6,7] In fact, the absence of B7-1 and B7-2 on most cells of peripheral organs, including the trophoblast cells in the placenta, may be important in maintaining immunological tolerance.[8,9] Consistent with this principle, cancer cells that are unable to effectively elicit an immune response can be rendered immunogenic by way of ectopic expression of either B7-1 or B7-2.[10,11] This was a key finding that has sparked intense investigation into the therapeutic potential of manipulating the B7-CD28 signaling pathway.

Two additional mechanisms involving CTLA-4 could also account for lymphocyte tolerance. First, delivery of the CTLA-4 signal, which inhibits production of interleukin (IL)-2 transcription and cell cycle progression, may be important for actively inducing T cell nonresponsiveness.[12] The second pertains to the recent finding that B7s can "back-signal" into antigen presenting cells. Using B7-1/B7-2 double-transfectants, Grohmann et al.[13] showed that ligation of these molecules by soluble CTLA-4 induces the production of indoleamine 2,3-dioxygenase, which catabolizes tryptophan and thus starves T cells of this essential amino acid. This enzyme has been shown to be essential for survival of fetuses in allogeneic murine pregnancy.[14]

Ober and coworkers were the first to propose a role for CTLA-4 in maternal-fetal tolerance. They found that inheritance of maternal alleles containing long stretches of AT repeats in the 3' untranslated region of the gene is associated with unexplained recurrent spontaneous miscarriage.[15] Since this microsatellite polymorphism could lead to decreased mRNA stability, it was hypothesized that aberrant or reduced expression of CTLA-4 at the maternal-fetal interface might compromise immunological protection of the fetus. Soon it was realized that CTLA-4 protein is expressed by fetal fibroblasts within the placenta.[16] As there are very few examples of CD28 family molecule expression outside of the lymphoid lineage the possible function of CTLA-4 expression in placental fibroblasts was a conundrum. Recently, however, we found that B7-2 is expressed by Hofbauer cells.[8] Together with the most recent observation that these cells also produce IDO,[17] CTLA-4 expression by placental fibroblasts now takes on significance. Given the findings of Grohmann et al,[13] it is possible that ligation of B7-2 on macrophages by CTLA-4 on fibroblasts induces IDO production by the macrophages, establishing the production of this critical immunosuppressor within the placental villous core.

B7-H1 and B7-DC

B7-H1[‡], the third B7 family molecule to be discovered, was identified by screening expressed sequence tag (EST) libraries for genes having amino acid sequence homology to B7-1 and B7-2.[18,19] Expression of these genes in the placenta was inherently obvious, as the studies reported cloning of the full-length cDNA for B7-H1 from human placental cDNA libraries. The 290 amino acid sequence of B7-H1 encodes a leader sequence, an IgV receptor-binding domain, an IgC domain, a transmembrane region, and a short cytoplasmic domain. This tertiary amino acid structure is similar to B7-1 and B7-2.

Shortly after the identification of B7-H1, a second new member of the B7 family was reported, called B7-DC.[20,21] B7-DC shares 38% amino acid identity with B7-H1, and the two proteins share a receptor. Like B7-H1, B7-DC possesses extracellular IgV and IgC domains, a transmembrane region, and a cytoplasmic tail. The B7-DC mRNA can be processed into three alternatively spliced variants, including one that lacks the IgV domain, one that lacks the IgC

[‡] Many of the newly identified members of the B7 family protein were discovered independently by two or more investigators, and thus are known under multiple aliases, as detailed in Table 1. For simplicity, we will utilize the nomenclature whereby homologues of B7 proteins are known as B7-*, where * is the suffix attributed to B7 family members, distinct from B7-1 and B7-2.

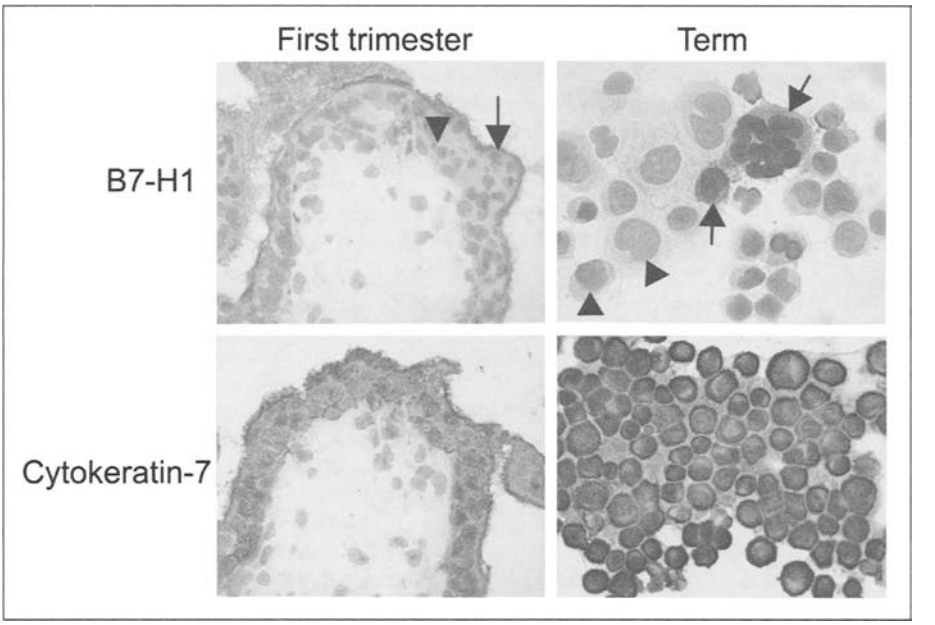

Figure 1. B7-H1 expression in first trimester and term villous placenta. Arrowheads indicate villous trophoblast precursor cells, while arrows indicate syncytiotrophoblast (first trimester) or syncytiotrophoblast fragments from purified cytotrophoblast cells (term). Cytokeratin-7 staining is shown to demarcate cytotrophoblast populations.

domain, and a possible soluble isoform that lacks both the IgC and transmembrane/cytoplasmic domains.[20,22] The significance of these alternatively spliced messenger RNAs and whether they are translated in vivo is not yet known.

Upon examination of multiple tissues by Northern blot panels, both B7-H1 and B7-DC messenger RNAs were found to be broadly expressed in a variety of tissues, suggesting that they have a role in regulating the immune response in local microenvironments. In fact, placental B7-H1 mRNA expression is very strong relative to other tissues, suggesting an exceptional need for this gene in the placenta.[18,20] On the other hand, the relatively widespread mRNA distribution is not necessarily reflected in similar distribution of the proteins. While the presence of B7-H1 in several fetal tissues has been reported,[23] others have shown that it is undetectable by immunohistochemistry in the parenchyma of many normal adult tissues.[24] Instead, constitutive expression of B7-H1 protein appears to be primarily limited to resident tissue macrophages and possibly dendritic cells. B7-DC protein expression may also be limited, although only a limited number of adult tissues have as yet been examined.[23,25]

In marked contrast to the restricted expression of the protein in most tissues, both B7-H1 and B7-DC are highly expressed in the human placenta.[8,23,26] While B7-H1 is predictably expressed by decidual macrophages, it is also strikingly abundant in all populations of trophoblast cells throughout gestation. In the syncytiotrophoblast, B7-H1 is particularly abundant at the microvillous surface, facing maternal blood (Fig. 1). On the other hand, B7-DC expression is localized to endothelial cells and possibly perivascular cells of the term placenta (Fig. 2).[23] Thus, B7-H1 protein in the placenta is specifically positioned at the interface between fetal and maternal blood and tissues, while B7-DC appears to be more restricted to the villous mesencymal core. The cell-specific expression of B7-H1 and B7-DC strongly suggests that these proteins are regulated by different mechanisms and perform nonoverlapping functions.

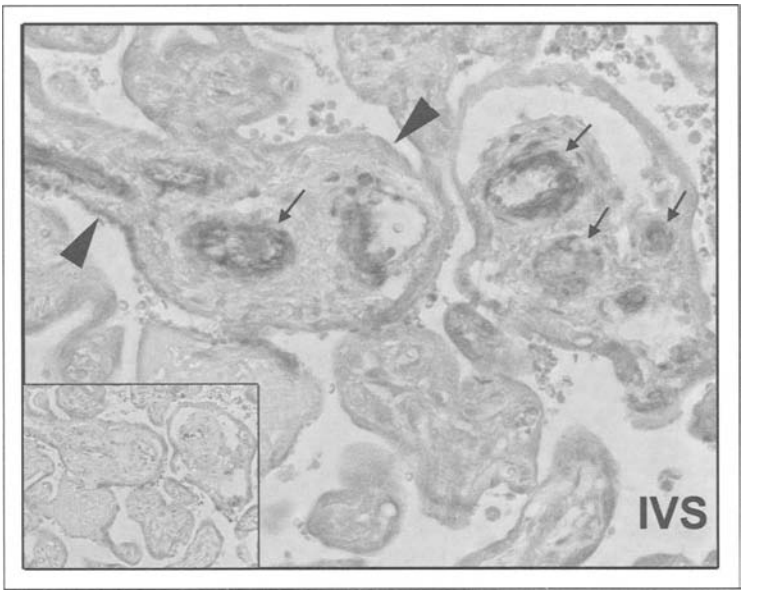

Figure 2. B7-DC expression in the term placenta. Arrows show positively stained placental blood vessels; arrowheads show unstained syncytiotrophoblast. IVS, intervillous space; inset, adjacent section stained with goat IgG.

While little is as yet known about regulation of in B7-DC in the placenta, B7-H1 abundance varies both spatially within the placenta and temporally across gestation, suggesting that expression of the protein can be fine-tuned according to the needs of trophoblast subpopulations at different gestational stages. Specifically, there is a link between exposure of trophoblast cells to maternal blood lymphocytes and elevated B7-H1. Two observations support this idea. First, during the first trimester, when maternal blood flow to the placenta is incomplete, the overall level of B7-H1 in the placenta is lowest.[8,27] In contrast, B7-H1 levels are much higher in the second and third trimesters, when the placenta is highly perfused with maternal blood. The increase in B7-H1 protein expression may occur at or near the time of onset of exposure to maternal blood, and therefore maternal lymphocytes, suggesting that the placental requirement for B7-H1 protein is elevated in the presence of maternal lymphocytes.

The second finding is that the syncytiotrophoblast, which lies in direct apposition to maternal blood and lymphocytes, expresses higher B7-H1 than the underlying cytotrophoblast precursor cells, which normally are not exposed to maternal blood (Fig. 1). Mere exposure to maternal blood may not be the defining factor in induction of B7-H1 protein, since in vitro differentiation of cytotrophoblast cells recapitulates in vivo expression patterns: treatment of purified term cytotrophoblast cells with epidermal growth factor results in increased expression of B7-H1.[8] Therefore, differentiation of trophoblast cells may be integrally linked to upregulation of B7-H1. Thus, multiple mechanisms may be in place within cytotrophoblast cells to ensure the induction of B7-H1 protein upon exposure of fetal tissues to maternal blood.

In addition to EGF and factors associated with increased blood delivery to the placenta, B7-H1 may be regulated in the placenta by interferon-γ. Both Jeg3, a choriocarcinoma cell line, and primary trophoblast cells respond to IFN-γ rapidly in terms of elevated B7-H1 mRNA and protein expression. IFN-γ, which appears to be critical for normal pregnancy,[28] may indirectly bring about immunosuppression by inducing mechanisms to offset immune responses, despite its notoriety as a proinflammatory cytokine.[29] Finally, evidence to suggest

that differential regulation of B7-H1 and B7-DC occurs in response to Th1 and Th2 cytokines, the balance of which may be critical in determining pregnancy outcome, has recently been reported.[25,30,31]

Many studies point to an immunoinhibitory role for B7-H1 and B7-DC acting through their receptor, PD-1, which was first described more than 10 years ago as a marker on activated T cells.[19,20,32] While not present on resting T cells, PD-1 is rapidly upregulated after activation; it can also be expressed on IgM- and IgM + CD40-stimulated B cells and dendritic cells.[33,34] PD-1 is a member of the CD28 family and, like CD28, it possesses a single extracellular IgV domain. Additionally, the intracellular domain contains an intracellular tyrosine inhibitory motif (ITIM) and an intracellular tyrosine switch motif (ITSM).[32,35,36]

In vitro studies have revealed that B7-H1/B7-DC signaling through PD-1 suppresses proliferation and cytokine production by activated T cells.[19,20,37] Roles for PD-1 in both allograft survival and immune evasion by tumor cells have also been suggested.[24,38,39] In addition, through a receptor that may be distinct from PD-1, B7-H1 signaling may promote T cell apoptosis through an IL-10- and FasL-dependent pathway;[24] similar mechanisms of deleting potentially harmful T cells during pregnancy have been suggested.[1,40,41] This raises the possibility that ligation of trophoblast-specific B7-H1 stimulates the Fas/FasL and IL-10 pathways, ensuring that lymphocytes potentially reactive to trophoblasts are eliminated. Finally, through a mechanism not yet well understood, B7-H1 and B7-DC may also serve as costimulatory molecules to T cell activation.[18,21]

In vivo studies have shown that the primary function of the B7-H1/-DC/PD-1 pathway is to maintain lymphocyte tolerance to self antigens. In PD-1-deficient mice, peripheral lymphocytes cells that would normally be deleted instead become activated and self-destructive.[42,43] Pathologies are marked by tissue specific antibody and complement deposition on affected organs.[42-44] This finding may be particularly relevant to placental immunology in light of the fact that complement deposition in the early placenta is lethal to pregnancy.[45] In addition, targeted deletion of B7-H1 in mice results in exacerbated susceptibility to autoimmune disease.[46,47] Neither of the latter reports noted alterations from expected Mendelian frequencies in the birth of B7-H1-/- pups, suggesting that B7-H1 is not a prerequisite for fetal survival in the mouse. However, a detailed analysis of B7-H1-deficient, B7-DC-deficient, and B7-H1/B7-DC double deficiencient mice will be required to fully evaluate the contribution of these molecules to immunotolerance in pregnancy.

B7-H2

B7-H2 has been identified by several independent groups, and thus has several aliases (Table 1).[48-51] B7-H2 has a tertiary structure similar to other B7 family proteins. The protein is constitutively expressed on B cells and can be induced on fibroblasts and nonlymphoid tissues by tumor necrosis factor-α.[48] The mRNA for B7-H2 is broadly expressed, suggesting that, like B7-H1 and B7-DC, B7-H2 functions to regulate local immune responses. There is at least one alternatively spliced transcript of B7-H2, called GL50, which differs in the C-terminal 10 amino acids.[52] The functional differences between B7-H2 and GL50 are not yet clear, but the two appear to have equally effective in promoting anti-tumor activity.[53] Of the two splice variants, B7-H2 mRNA is the more broadly distributed.[51]

B7-H2 is the ligand for ICOS (Inducible COStimulator of T cells), another CD28 family molecule. ICOS is expressed by activated and resting memory T cells.[49] Evidence has steered researchers towards the hypothesis that the B7-H2 – ICOS pathway preferentially directs Th2 cell effector functions. While ICOS does not costimulate the production of the Th1 cytokine IL-2, it does costimulate IFN-γ and IL-4 production.[54] Notably, it is a strong inducer of IL-10 production.[54] During T cell activation, ICOS expression is induced early; however, only T cells that differentiate into the Th2 subset retain ICOS expression on the cell surface.[55] In vivo, the level of surface ICOS expression is strongly correlated with the degree of Th2 cytokine production, and blockade of ICOS during stimulation of T cells in vitro polarizes the cells towards

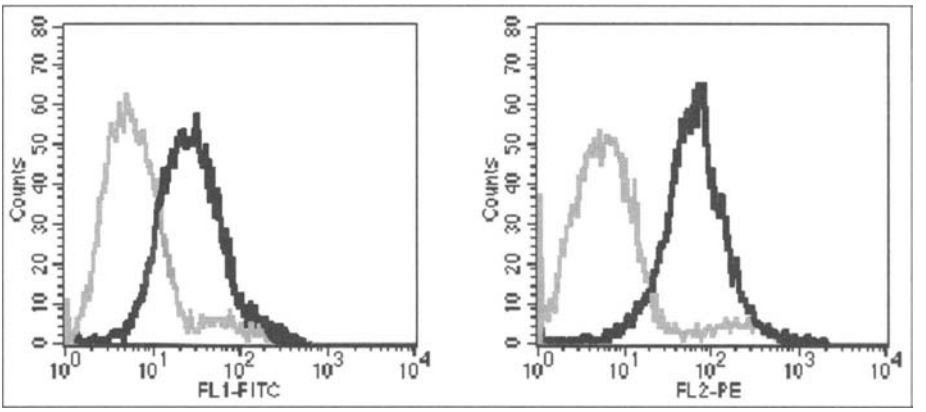

Figure 3. B7-H1 and B7-H2 expression by the Jeg3 choriocarcinoma cell line. Left panel, B7-H1 (black line) and mouse IgG (grey line); right panel, B7-H2 (black) and mouse IgG (grey).

Th1 cytokine production.[55,56] Recent findings also suggest that ICOS signaling plays a unique role in controlling clonal expansion of differentiated Th2 cells.[57]

Ling and coworkers[51] first suggested that the presence of GL50 transcripts in the murine embryo-derived yolk sac serves to skew maternal cytokines towards the Th2 profile. B7-H2 mRNA and protein are present in the human and murine placenta, (Petroff et al, unpublished observations). In addition, we have found that, like B7-H1, B7-H2 is expressed on the surface of the Jeg3 cell line, suggesting that this protein is present on cytotrophoblast cells (Fig. 3).

Interestingly, ICOS can be detected on natural killer cells, which are abundant at the maternal-fetal interface in both humans and mice. IFN-γ, which is produced by the placenta and by uterine NK cells,[28,58] upregulates ICOS expression in NK cells. ICOS cross-linking enhances the cytotoxicity of these cells towards tumor cells.[59] These findings pose an apparent conundrum as to the possible function of B7-H2 at the maternal fetal interface. However, a detailed analysis of the distribution of both of these molecules will provide invaluable clues as to their function in situ.

B7-H3

B7-H3 was first cloned from a dendritic cell-derived cDNA library.[60] Analysis of the cDNA initially projected B7-H3 to have a 316 amino acid sequence containing a signal peptide, an extracellular domain consisting of one IgV and one IgC domain, a transmembrane domain, and a 45-amino acid cytoplasmic tail. Subsequent reports revealed additional information about the B7-H3 gene.[61-63] Within the region encoding the extracellular domain, the human B7-H3 gene is constructed of two IgV and two IgC domain exons, in a tandemly duplicated, "$V_1C_1V_2C_2$" arrangement. Thus, the B7-H3 gene is alternatively spliced into two known isoforms: a 534 amino acid, four Ig domain protein with an extracellular region of $V_1C_1V_2C_2$, and a shorter 316 amino acid, two Ig domain protein with an extracellular arrangement of V_1C_2. In contrast, the rodent gene contains exons for single IgV and IgC domains, and pseudo (Ψ) V and C domains that are not transcribed. The gene has a "$V_1C\Psi V\Psi C_2$" organization, and is transcribed only as one isoform, "V_1C_2".[63]

Multiple tissue Northern blots showed that, like the other newly discovered members of this family, B7-H3 is broadly expressed by tissues, including the placenta.[60-62] The absence of B7-H3 mRNA in peripheral blood lymphocytes confirms that its presence in the placenta is not simply due to contaminating blood.[63] The transcript corresponding to the four-Ig B7-H3 protein predominates in all tissues examined. Interestingly, one group reported that only the

less abundant two-Ig molecule is present in the placenta,[62] suggesting that there could be individual variation, or alternatively, placenta-specific processing of this gene.

The counter-receptor for B7-H3 has not yet been identified. However, using a B7-H3/Fc fusion protein, the putative receptor for B7-H3 was found to be transiently present on activated, but not resting $CD4^+$ and $CD8^+$ T cells.[60-62] The first description of B7-H3 was as a costimulatory molecule that enhances T cell proliferation and augments killer activity of cytotoxic T lymphocytes.[60] An 18-fold enhancement of IFN-γ production by target T cells was also reported. In vivo, B7-H3-expressing tumors were either eradicated or slower growing than the nonB7-H3-expressing controls, further suggesting that B7-H3 promotes antitumor immunity in a CTL or NK cell-dependent manner.[62] On the other hand, in findings reminiscent of the dual functions of B7-H1 and B7-DC, other investigators have found that B7-H3 can inhibit T cell activity. In these studies, both the long and short forms of B7-H3 were able to inhibit T cell proliferation, as well as production of the cytokines IL-2, IL-10, TNF-α, IFN-γ, and GM-CSF.[63,64]

Mice containing a targeted deletion of the B7-H3 gene were prone to developing exaggerated Th1-type immune responses, supporting an immunosuppressive and tolerogenic role for B7-H3.[64] Additionally, lymphocytes from these mice had a stronger ability to stimulate alloreactive T cells. However, despite the expression of B7-H3 in the placenta, B7-H3-deficient mice, like B7-H1 knockouts, did not exhibit gross developmental defects and were born of the expected Mendelian frequency, suggesting that placental expression of B7-H3 is not required for fetal survival.[64] However, it will be important to undertake studies that examine the reproductive and fertility aspects of these mice in detail, as well as under different environmental conditions.

B7-H4

B7-H4 is the seventh and most recently discovered B7 family members.[65-67] Like most other B7s, B7-H4 contains within its extracellular region one IgC domain and one IgV domain. However, B7-H4 is unique in that it is associated with the cell membrane through a glycophosphatidylinol (GPI)-linkage.[67]

BTLA (B and T Lymphocyte Attenuator) is the sole known receptor for B7-H4.[66] As with most other CD28 family members, BTLA is not expressed on the surface of resting lymphocytes, but is instead induced upon activation. An important feature of BTLA appears to be its preferential expression on lymphocytes that are polarized toward the Th1 phenotype. Thus, whereas ICOS appears to play an important role in Th2 immune responses, BTLA may be particularly relevant to the controlling Th1 activity or cytokine production.

B7-H4 has consistently been shown to suppress activation of both $CD4^+$ and $CD8^+$ T cells by means of inhibiting of IL-2 production.[65,67,68] In in vitro assays, the inclusion of CD28 crosslinking antibody can greatly enhance TCR-mediated stimulation by anti-CD3 antibody. Similar to the effects of B7-H1 and B7-DC, B7-H4 partially inhibited CD3 and CD28-mediated T cell proliferation, suggesting that the T cells are less responsive to these stimuli in the presence of inhibitory B7 family molecules. In vivo, inhibition of B7-H4 function using blocking antibody or genetic targeting exacerbated the development of experimental autoimmune encephalitis in mice, causing enhanced infiltration of autoreactive T cells and macrophages into the brain.[66,67]

While B7-H4 mRNA is also broadly expressed in many tissues. In the placenta, B7-H4 mRNA was detected at high levels by northern blot analysis in one study, but was detectable only by RT-PCR in another; thus, the abundance of B7-H4 in the placenta is as yet uncertain.[65,67] Even so, in our hands B7-H4 mRNA expression is reproducibly detected by microarray analysis of highly purified preparations of cytotrophoblast cells (Petroff, unpublished results). Thus, there is little doubt that the B7-H4 gene is expressed in the placenta and indeed, in cytotrophoblast cells. Given the proposed role in suppressing Th1 immunity, expression of B7-H4 in trophoblast cells would likely also support immunoprotection in the placenta.

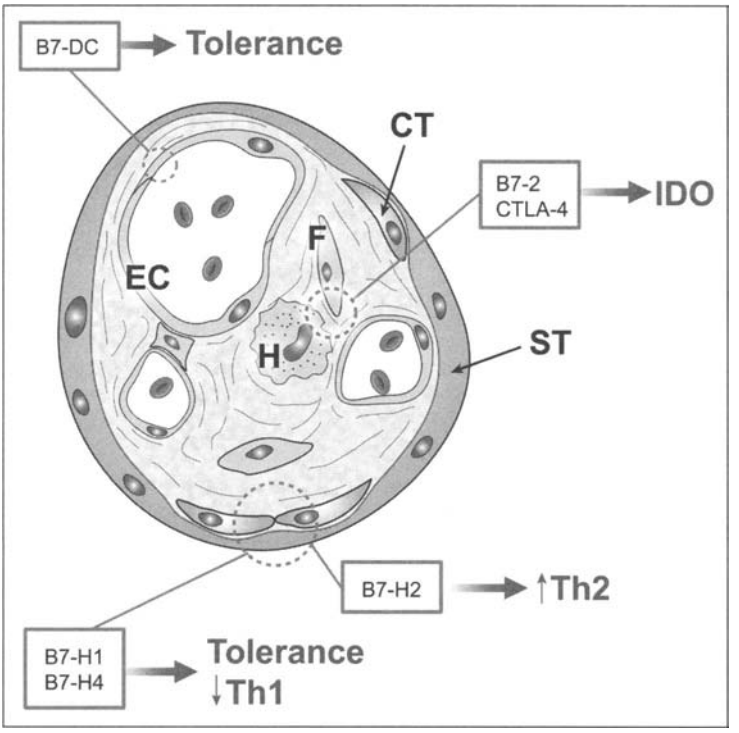

Figure 4. Summary of expression and potential function of B7 family proteins in the placenta. Shown is a schematic diagram of a cross section of a term placental villus. Abbreviations: EC, endothelial cell; H, Hofbauer cell; F, fibroblast; CT, cytotrophoblast; ST, syncytiotrophoblast; IDO, indoleamine 2,3-dioxygenase.

Summary and Conclusions

Figure 4 summarizes the expression patterns and possible functions of B7 family molecules in the term placenta. The CD28 family member CTLA-4, which is expressed by placental fibroblasts, may be in position to interact with B7-2 on Hofbauer cells to result in production of IDO. B7-H1 is expressed by the syncytiotrophoblast and other populations of trophoblast cells, and may bind PD-1 on the surface of lymphocytes in the peripheral blood or decidua, thereby inhibiting there activation and inducing tolerance. Intriguingly, a population of regulatory T cells that is important in both transplantation tolerance and pregnancy success expresses PD-1, suggesting that another function of B7-H1 expression on trophoblast cells is to regulate these cells.[69-71] Similar immunoprotective and tolerogenic roles may be played by B7-H3 and B7-H4; examination of their expression patterns will give further clues as to their possible function. B7-DC, on the other hand, localizes to the endothelial cells of placental blood vessels; here, its role could be to interact with developing fetal lymphocytes, perhaps inducing fetal self-tolerance. Collectively, the expression of B7-H1, -H3, and -H4 may be an important means by which trophoblast cells and other placental cells signal or mimic self-identity in order to subvert immunological attack. Lastly, B7-H2, a key mediator of the Th1/Th2 balance, could induce or preserve placental maintenance of the immunoprotective Th2 cytokine bias.

Future studies detailing the expression patterns of the B7 ligands and their CD28 receptors at the maternal-fetal interface, as well as examination of genetic polymorphisms of these genes in relation to pregnancy-associated disorders such as preeclampsia and recurrent spontaneous miscarriage, will provide additional important clues as to the function of these proteins.

Ultimately, in vitro assessment of the functional consequences of trophoblast expression of B7 family proteins and in vivo analysis of gene-targeted B7-deficient mice will provide the best evaluation of their importance in the placenta.

Acknowledgements

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CHAPTER 16

The Role of Regulatory T Cells in Materno-Fetal Tolerance

Varuna R. Aluvihare and Alexander G. Betz

Maternal immune tolerance to paternal allo-antigens expressed by the fetus is a precondition of successful pregnancy in viviparous mammals. This occurs despite exposure of the maternal immune system to potentially immunogenic fetal tissue. Local immune evasion mechanisms are thought to prevent maternal immune aggression towards the fetus. Nevertheless, maternal lymphocytes capable of mediating anti-fetal immune aggression are detectable throughout gestation. This review focuses on the role played by regulatory T cells in suppressing alloreactive immune responses, thereby promoting materno-fetal tolerance. The characteristics of naturally occurring regulatory T cells, their function in mouse and human pregnancy and the implications for infertility, premature abortion syndromes, transplantation and autoimmunity are discussed in detail.

Thymic positive and negative selection generates a lymphocyte repertoire, which enables the immune system to attack foreign antigen (such as invading pathogens), whilst ignoring self-antigen; failure of this tightly regulated process can lead to immune deficiency and autoimmunity respectively.[1] Whilst solid organ transplants with allotypic differences at major histocompatibility loci (MHC class I and II) are recognized as foreign and rejected,[2] the maternal immune system does not attack the fetus despite expression of paternally derived allo-antigens. This constitutes an immunological paradox, which has fascinated immunologists for decades. Whilst the mechanisms that ensure materno-fetal tolerance usually prevail, maternal immune aggression is increasingly recognised as a cause for premature termination of pregnancy.

Peter Medawar, in his pioneering research into materno-fetal tolerance, hypothesised three mechanisms that may confer immune protection to the fetus.[3] He proposed that the fetal and maternal circulations are segregated such that the maternal immune system has no access to fetal tissue. He also proposed that fetal tissue might be immunologically immature, thereby failing to elicit maternal immune responses. Finally, he proposed that the maternal immune system might somehow ignore potentially immunogenic fetal tissue. It is now evident that fetal cells gain access to the systemic maternal circulation and that fetal tissue is immunologically mature with regard to the expression of MHC class I and class II.[4] Research has therefore focused on the final hypothesis of maternal inertness to fetal antigen.

Although the fetus is frequently described as a semi-allograft, a number of significant differences raise questions regarding the validity of its comparison to an organ transplant. These include the highly specialized anatomy of the materno-fetal interface and the impact of hormonal changes prevailing during pregnancy on the maternal immune system.

Mechanisms Mediating Fetal Immune Evasion

Rather than ignoring the fetus as Medawar hypothesised, the maternal immune system in fact responds to fetal allo-antigens.[5,6] Therefore, a number of fetal evasion strategies have evolved

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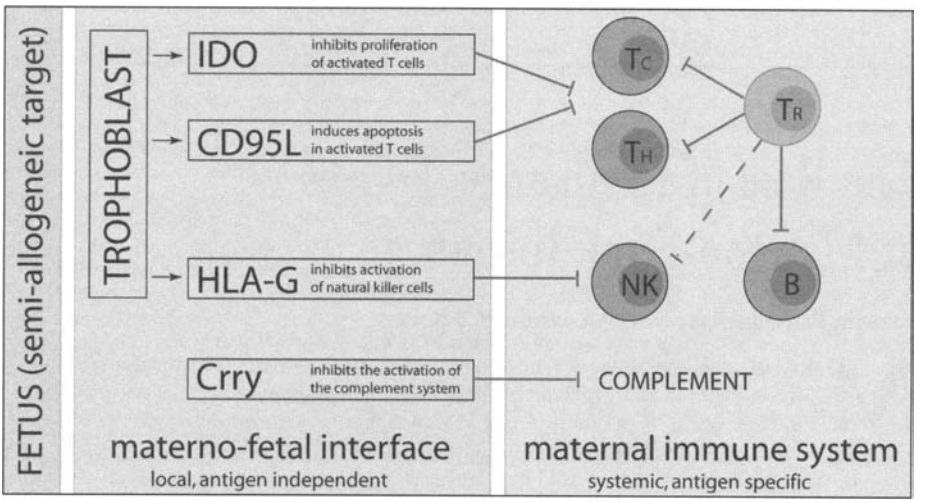

Figure 1. Local, antigen independent versus systemic, antigen specific mechanisms of materno-fetal tolerance. Fetal trophoblasts express a variety of molecules which mediate a local, antigen independent immune suppression (highlighted in pink). IDO and CD95L promote cell death of allo-reactive maternal helper T cells (T_H) and cytotoxic T cells (T_C); HLA-G inhibits natural killer cells (NK); Crry inhibits maternal complement activation. Systemic and local immunosuppression of allo-responses (highlighted in green) is mediated through inhibition of maternal helper T cells (T_H), cytotoxic T cells (T_C), B cells (B) and possibly natural killer cells (NK) by regulatory T cells (T_R). To view color figure, go to http://www.eurekah.com.

that act locally to either kill activated maternal lymphocytes or inhibit the activation of effector pathways that lead to tissue damage (Fig. 1).

In a series of elegant studies, Mellor and Munn showed that the syncytiotrophoblast, the specialized fetal tissue which invades the uterus, synthesises and secretes Indoleamine 2,3-dioxygenase (IDO).[7] IDO function locally depletes tryptophan, which in turn kills rapidly dividing maternal effector lymphocytes.[8] Inhibition of IDO function has been shown to lead to immunological rejection of allogeneic fetuses in the mouse. Further, trophoblast tissue expresses the death promoting Fas ligand (CD95L), a member of the tumor necrosis factor (TNF) family.[9] Activated maternal lymphocytes, which express the receptor Fas (CD95), will apoptose when they come in to contact with trophoblast tissue expressing Fas ligand. Trophoblast tissue also expresses the nonpolymorphic human leukocyte antigen (HLA) HLA-G, whilst the polymorphic HLA-A, HLA-B and HLA-C are barely, if at all, detectable.[10] This results in inhibition of the cytotoxicity of natural killer cells (NK cells), which are the dominant mononuclear cells infiltrating the uterus. The initiation of the complement cascade by maternal lymphocytes recognizing fetal alloantigen would be deleterious to the fetus. Expression of the complement regulator protein Crry prevents such activation.[11] Lack of Crry expression leads to complement deposition at the materno-fetal interface and gestational failure. Finally, leukaemia inhibitory factor (LIF), which is secreted by the endometrium during implantation, appears to promote materno-fetal tolerance since reduced levels are associated with pregnancy loss in humans.[12]

Although these mechanisms contribute to deletion of anti-fetal effector cells, such cells clearly survive during gestation. In a pivotal study by Tafuri et al, pregnant mice were able to tolerate systemic engraftment of paternally derived tumour cells, although more immunogenic grafts were rejected (Fig. 2).[5] Maternal tolerance was restricted to the duration of gestation. This work not only highlights the specific tolerance to paternal allo-antigens engendered in the maternal immune system, but the persistence of potentially aggressive anti-fetal effector cells throughout gestation. In addition, the local deletion mechanisms described above cannot

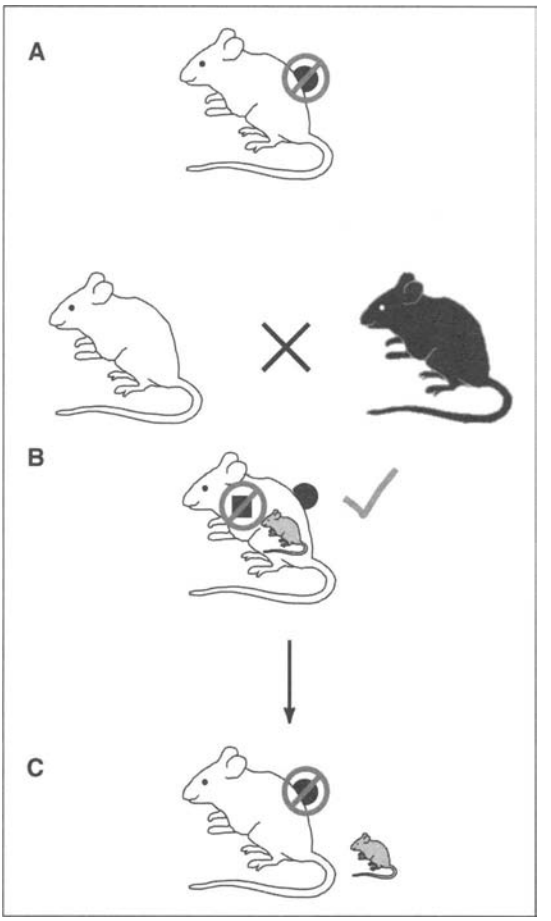

Figure 2. Pregnancy induces specific maternal tolerance to paternal alloantigens despite persistence of alloreactive lymphocytes. A) In nonpregnant mice allogeneic tumours (black circle) are readily rejected. B) In pregnant mice carrying semi-allogeneic fetuses, paternal tumours are tolerated until, C) delivery. Despite this, more immunogenic, allogeneic skin grafts (black square) are rejected. This highlights the systemic persitance of alloreactive maternal lymphocytes, which have escaped local deletion mechanisms. The tolerance to paternal tumours suggests that these allo-reactive cells are at least partially regulated.

explain the amelioration of a number of autoimmune diseases during pregnancy in both mice and humans.[13,14]

We hypothesised that regulatory T cells may contribute to materno-fetal tolerance by suppressing anti-fetal responses and contribute to the systemic changes which occur during pregnancy.

Markers and Characteristics of Regulatory T Cells

The demonstration by Billingham and Medawar that neonatal transfer of cells could induce tolerance in adult mice initiated a body of research aimed at delineating the mechanisms mediating tolerance.[15] While negative selection in the thymus (central tolerance) undoubtedly plays a major part in promoting self-tolerance, systemic auto-reactive cells are readily detectable in normal healthy mice and humans.[1] In vitro and in vivo experiments demonstrated the existence of cells capable of suppressing such potentially deleterious, auto-reactive cells (peripheral tolerance). However, at the time, further attempts to characterise the mechanisms that mediate peripheral, active suppression were hindered by the lack of markers for these 'suppressor cells'. The rise, fall and subsequent reemergence of T cell suppression, leading to the characterisation of regulatory T cells is beyond the scope of this review. However, the characteristics and function of naturally occurring regulatory T cells is outlined below.

The current resurgence of interest in regulatory T cells resulted in no small part from the pioneering experiments of Sakaguchi and colleagues, who showed that neonatal thymectomy between day 2 and 4 resulted in organ specific auto-immunity.[16] By this stage, previously generated mature thymocytes have already emigrated to the periphery. Transfer of cells from these mice into mice lacking any T cells (nude mice) caused similar disease. In both instances, transfer of cells from normal adult mice could suppress disease. These experiments highlight the fact that the neonatal thymus generates naturally occurring regulatory T cells from day 2. These cells were subsequently characterised to be $CD4^+CD25^+$ cells. Other markers that characterise these cells include the surface expression of glucocorticoid induced TNF receptor family-related gene (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and transforming growth factor β (TGFβ), as well as soluble TGFβ and interleukin 10 (IL-10) production.[17] Stimulation of GITR on regulatory T cells appears to abrogate the suppressor function of these cells and antibody mediated stimulation in vivo leads to organ specific autoimmune disease.[18] Interaction of GITR with its ligand may therefore constitute a mechanism for terminating regulatory T cell function during an immune response. Regulatory T cells are typically anergic, in that they do not proliferate in response to T cell receptor triggering in vitro, unless additionally stimulated with IL-2.[17] However, since none of these characteristics are unique to regulatory T cells, it has proved difficult to exclusively manipulate these cells in vitro or in vivo to study their function.

The antigen specificity of regulatory T cells has been the focus of much research. Experiments with double transgenic mice expressing a T cell receptor transgene as well as its cognate antigen, indicate that regulatory T cells are thymically selected for self-antigen/MHC.[19] Positive selection for regulatory T cells appears to occur at affinities, which in nonregulatory T cells are thought to lead to deletion by negative selection. Transgenic experiments in which cognate antigen expression was driven by different promoters, indicates that antigen expression by thymic epithelial cells drives selection of naturally occurring regulatory T cells.[20]

Regulatory T Cell Function

In vitro, regulatory T cells can inhibit the proliferation or function of dendritic cells, naive $CD4^+$ T cells, $CD8^+$ T cells[17] and B cells.[21] Although the mechanism of action is not entirely understood, using a variety of experimental models, CTLA-4, TGFβ and IL-10 have all been implicated. These experiments also highlight the fact that regulatory T cells are activated in an antigen specific manner, but can subsequently suppress responses to unrelated antigens (linked suppression).

In vivo, regulatory T cell function has been explored using CD25-antibody mediated depletion and adoptive transfer experiments of lymphocytes into lymphopenic (*nu-/nu- or rag-/-*) hosts after CD25 T cell depletion. Similar adoptive transfer experiments have been carried out using CD45RB expression to distinguish regulatory T cells from naïve $CD4^+$ T cells.[22] In these studies, $CD4^+$ T cells expressing low levels of CD45RB have all the characteristics of naturally occurring regulatory T cells. In the absence of regulatory T cells, mice develop a multitude of organ specific autoimmune diseases, which could be rescued by transfer of purified regulatory T cells. In spite of these findings and the in vitro evidence of suppressor function, concerns remained that regulatory activity was the result of the highly manipulated systems used to study these cells. These have largely been addressed by the recent description of a unique lineage marker, the fork-head transcription factor Foxp3.[23-25] Forced expression of Foxp3 in naive $CD4^+CD25^-$ T cells in vitro confers some aspects of regulatory T cell function. Mutations in this gene, in both mice (scurfy) and humans (IPEX syndrome), leads to regulatory T cell deficiency and overwhelming autoimmune disease. These findings have confirmed the pivotal role of regulatory T cells in maintaining peripheral tolerance.

As well as suppressing organ specific autoimmune disease, regulatory T cells have been implicated in mediating tolerance to organ transplants.[2] Deleterious effects of regulatory T cell function include the promotion of chronic infections and tumour recurrence.[26] Interestingly,

regulatory T cells inhibit pathogen clearance in murine cutaneous leishmaniasis and thereby promote immunity to reinfection.[27]

Other Cells with Regulatory Function

In addition to the naturally occurring regulatory T cells described above, other cells with regulatory or suppressor capability have been described. Murine and human, $CD4^+$ Tr1 cells are generated by stimulation with IL-10 and antigen.[28] As such they represent an inducible lineage in contrast to the naturally occurring regulatory T cells. Tr1 cells are generated extra-thymically, characteristically secrete IL-10 and can suppress antigen specific immune responses in vitro and in vivo. Confusingly, as these cells can be both CD25 positive and CD25 negative, they are not readily distinguishable from the regulatory T cells described above. Although their relationship to naturally occurring regulatory T cells remains unclear, there is little convincing evidence to suggest that these cells express Foxp3.

In addition, under some experimental conditions $CD8^+$ T cells, $\gamma\delta$T cells and NK cells have been shown to possess immunosuppressive capability.[28] It is also noteworthy that dendritic cells (DC) are not only potent initiators of immune responses, but under some circumstances appear capable of stimulating regulatory T cells. In mice, antigen stimulated bone marrow derived DC can stimulate and expand $CD4^+CD25^+$ regulatory T cells in vitro and in vivo.[29] Although these cells were capable of suppressing activation of $CD25^-$ T cells in vitro, it is not clear in this study whether the $CD4^+CD25^+$ T cells are naturally occurring or Tr1 like cells. Human DC on the other hand, can induce inflammatory responses or stimulate IL-10-producing Tr1 cells, depending on their state of maturation.[30] The capacity of DC to stimulate regulatory T cells raises the question of how invading pathogens initiate immune responses. Toll-like receptors recognise conserved pathogen associated molecular patterns (like lipopolysaccharide and CpG) and thereby detect the presence of infection. Stimulation of murine immature splenic DC with TLR ligands promotes their maturation.[31] This leads to an IL6 dependent inhibition of suppression mediated by $CD4^+CD25^+$ regulatory T cells and activation of pathogen specific adaptive immune responses.

Regulatory T Cells Mediate Maternal Tolerance to the Fetus

Recent research highlights the role of regulatory T cells in mediating maternal tolerance to the fetus (Fig. 1).[6] The proportion of $CD4^+CD25^+$ cells in the spleen, draining lymph nodes and blood of syngeneically (genetically identical parents) and allogeneically (genetically different parents) mated mice significantly increases during pregnancy. Interestingly, this suggests that expansion of maternal regulatory T cells is not driven by allo-antigen exposure. A small but consistent increase in $CD4^+CD25^+$ cells can be detected as early as 2 days after successful mating. This finding suggests that the expansion of the $CD4^+CD25^+$ cell pool commences around the time of implantation, the implications of which are discussed below.

In vitro, maternal lymphocyte proliferation in response to syngeneic and allogeneic paternal stimulator cells invariably increased upon depletion of $CD25^+$ cells. Therefore, during normal pregnancy, the $CD25^+$ population of cells has dominant regulatory function. A remarkably high proportion (up to 30%) of $CD4^+$ lymphocytes in the uterus of pregnant mice are $CD25^+$. This influx of regulatory T cells is reflected in a significant increase in uterine Foxp3 expression upon pregnancy. Therefore, whilst pregnancy induces a systemic expansion of regulatory T cells, these cells appear in addition to be either preferentially recruited or locally induced.

The pivotal role played by regulatory T cells in mediating materno-fetal tolerance is highlighted by the finding that absence of $CD25^+$ cells leads to a failure of gestation in allogeneically but not syngeneically mated mice. Whilst in most cases the failure of pregnancy appears to occur early in gestation, aggressive lymphocytic infiltration of resorbing fetuses and haemorrhages at the materno-fetal interface can be observed as late as mid-gestation. These are cardinal features of immunological rejection of the fetus. As mentioned above regulatory T cell expansion appears to be concurrent with implantation. In addition to their function in later pregnancy, this suggests a role for regulatory T cells in the early post-implantation phase. In order to test

this possibility, a more detailed analysis of the effects of regulatory T cell depletion in early pregnancy needs to be carried out.

Three recent studies indicate that regulatory T cells play a similar role during human pregnancy.[32-34] They demonstrate an increase in systemic and decidual $CD4^+CD25^+$ T cells during the first and second trimesters. These studies show the expanded population of $CD4^+CD25^+$ T cells are CTLA-4^+ $GITR^+OX40^+$ and most importantly Foxp3 expressing, all characteristics of naturally occurring regulatory T cells. In agreement with the findings in mice, these cells suppress the proliferation of $CD4^+$ T cells in vitro.

The role played by regulatory T cells in mediating materno-fetal tolerance is emphasized by the finding that the proportion of decidual regulatory T cells is significantly lower in tissue obtained from cases of spontaneous abortion as compared to induced abortions.[33] A failure of immunological tolerance has been postulated in preeclampsia, infertility and spontaneous abortion syndromes. Taken together, the findings in mice and humans suggest that regulatory T cell function may be compromised in all of these conditions.

Interaction of Regulatory T Cells with Fetal Immune Evasion Mechanisms

Although the findings described above indicate a more global change in the maternal immune system, the influence of regulatory T cells may be exerted in the draining lymph nodes or indeed, at the materno-fetal interface. In addition to direct inhibition of alloreactive immune responses, these cells are capable of influencing a number of fetal immune evasion mechanisms. The recent demonstration that regulatory T cells can induce tryptophan catabolism in dendritic cells via CTLA-4 dependent and independent mechanisms, suggests that they may be capable of inducing IDO activity in the syncytiotrophoblast.[35] This would lead to an inhibition of T cell and complement mediated anti-fetal immune aggression, which occurs when IDO function is inhibited. Regulatory T cells have also been shown to exert a cytokine dependent suppression of innate immune pathology, in a mouse model of bacteria triggered intestinal inflammation.[36] In this study, regulatory T cells were capable of inhibiting natural killer cell activation, although it is unclear whether this is a direct or indirect effect.

Implications of Pregnancy-Induced Regulatory T Cell Expansion

The expansion of the regulatory T cell pool during pregnancy appears to be independent of allo-antigenic stimulation and it is tempting to speculate that this is driven by the hormonal changes prevailing throughout gestation. It is noteworthy that oestrogen and progesterone are known to exert a variety of influences on the immune system.[14]

The increased size of the regulatory T cell pool and therefore the increase in regulatory activity during pregnancy may underlie the gestational amelioration of some autoimmune diseases, which occur in both mice and humans. Furthermore, collagen induced arthritis (in mice), rheumatoid arthritis and multiple sclerosis (both in humans) often relapse after birth.[14] The recent demonstration of reduced regulatory T cell frequency and function in patients with multiple sclerosis argues that disease improvement during pregnancy may be related to the concurrent expansion of this pool.[37]

The increase in systemic regulatory T cell function may also explain the specific, systemic maternal tolerance to paternally derived tumour cells, which is limited to the duration of pregnancy.[5] However, this does not necessarily imply an immuno-compromised state. Regulatory T cells appear to be activated by self-antigen/MHC in order to suppress autoimmune or, in this case, semi-allogeneic responses. Thus, they would not be expected to suppress responses to exogenous antigens.

Delineating the mechanisms that underlie the expansion of regulatory T cells during pregnancy and render these cells capable of mediating materno-fetal tolerance, has implications for therapeutic intervention in organ transplantation and autoimmune disease. Furthermore, it may permit the beneficial manipulation of regulatory T cells in metastatic malignancy and chronic infections, where they contribute to pathogenicity.

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CHAPTER 17

The Eutherian Fetoembryonic Defense System Hypothesis: An Update

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Abstract

All sexually reproducing organisms produce gametes that must be protected from immune challenge. Recent data indicates that the majority of the carbohydrate sequences that coat the murine zona pellucida are also upregulated on activated lymphocytes, and some participate in gamete binding. This overlap indicates that there may be a "species recognition system" (SRS) that is employed to identify both immune cells and gametes in the context of "species" rather than "self". In eutherians, histoincompatible progeny must also be protected from the maternal immune response. The composite data indicates that several glycoconjugates produced in the pregnant can modify immune responses in vitro. We have previously referred to these collective factors as the "eutherian fetoembryonic defense system" or eu-FEDS. Based on the available results, it is very likely that these glycoconjugates are utilizing specific carbohydrate sequences as functional groups to mediate these activities. Many different persistent pathogens and tumor cells also either mimic or acquire these carbohydrate functional groups, indicating that they may be able to evoke similar effects. We outline new data that clearly implicate glycobiological subterfuge in the pathological effects associated with infection with lentiviruses like HIV-1 and simian immunodeficiency virus (SIV). In addition, recent data showing that the uterine mucin CA125 and the major surface glycoprotein of HIV-1 (gp120) express the same N-glycans suggests a linkage to the pregnant uterus. Complete integration of this protective effect associated with eu-FEDS could also explain why the natural hosts of SIV (sooty mangabees and African green monkeys) are able to completely accommodate this virus without pathological effects. In summary, the requirements for accommodation of the germ cells and different stages of developing progeny could provide a very substantial "Achilles heel" for exploitation by pathogens and tumor cells. These more recent finding should provide a great impetus to further investigate the eu-FEDS hypothesis and its strong linkages to pathogenesis.

Introduction

Every organism that obligatorily employs sexual reproduction is derived from a unique combination of genes contributed in equal measure from both maternal and paternal sources. Therefore any abnormal process that interferes with the reproduction of an individual eliminates its potential genetic contribution to the species pool. The impact of this extremely powerful selective force should be considered by all biomedical scientists, regardless of their area of investigation, because of its ability to shape the ultimate destiny of a species.

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It is also apparent that all major organ systems within an individual must obey this reproductive imperative. This deference is abundantly evident in the endocrine and nervous systems of sexually reproducing animals. However, it is also apparent that the immune system must also tightly be regulated in all sexually reproducing organisms to enable reproduction.[1-3] In simple egg laying individuals, this system may only require the protection of the gametes and fertilized eggs from potential immune responses.

More than 50 years ago, Peter Medawar suggested that temporal and spatial suppression of the immune response must also occur within the pregnant uterus of eutherian mammals to protect histoincompatible progeny.[4] Therefore a very potent means of immune modulation must be evoked in these mammals to protect histoincompatible progeny during gestation. In addition, this effect must be tightly regulated in a temporospatial manner to circumvent global immune suppression of the maternal immune system at a time when it must protect both the parent and the offspring.[3]

It is within this context that we have made an effort to understand this protective effect so essential for reproduction. Our data suggests that the gametes may protect themselves by expressing specific carbohydrate ligands on their surface that can be recognized by immune effector cells. Eutherians evoke this tolerant state by presenting specific glycoconjugates in either soluble or cell surface associated form to immune effector cells. One of the major operating principles that will subsequently be discussed here is that the oligosaccharides linked to these glycoconjugates act as "functional groups" to enable these immunomodulatory effects.

The initial data supporting the existence of this protective system was obtained in the human model. Therefore we first referred to this paradigm for glycoconjugate mediated suppression as the "human fetoembryonic defense system" or hu-FEDS.[5] However, it later became apparent that this system likely extended to all eutherians and was therefore designated the "eutherian fetoembryonic defense system" or eu-FEDS.[2] As we will discuss, we believe that eu-FEDS itself likely originated in the fundamental immune recognition processes that protect the gametes and fertilized eggs in lower organisms.

It also became apparent that this ability of the reproductive system to evoke natural suppression of the immune response provides an ample opportunity for exploitation by pathogens. Therefore any pathogen that could integrate itself into the system that protects the gametes and developing progeny from immune response would gain an enormous advantage, coupling its survival to the reproductive imperative of its host. Misuse of this system by pathogens could be the most significant "Achilles heel" of a sexually reproducing organism's immune response. Therefore the eu-FEDS hypothesis also proposes an extremely strong link to pathogenesis.[2] Let us consider some of the specific issues here related to these protective systems and their association with pathogenesis.

In the Beginning: A Model for the Protection of the Gametes

Collectively, gametes can be thought of as the repository of all the genetic potential within a given species. In obligatory sexually reproducing organisms, they should be afforded extraordinary protection from immune responses, because without them there is no future for a given species. Logically, a "fail-safe" system of discrete recognition signals for immune effector cells should be presented on their surfaces to prevent any potential cell mediated responses directed against them.[6]

Humans and mice represent by far the preferred models for investigating the immune system. However, at this time more is known about the murine rather than the human immune response. The mouse is also the preferred mammalian model for reproductive studies because of its fecundity, short lifespan, low maintenance costs, and perhaps most importantly, its ability to be genetically manipulated.

The eggs of all mammals including humans are coated with a specialized extracellular matrix known as the zona pellucida (ZP).[7] Glycoproteins associated with the murine ZP (mZP) have been extensively characterized, primarily because of their functional role in mediating

sperm binding and the induction of the acrosome reaction.[8,9] Overall, there does not appear to be considerable primary sequence homology between mZP glycoproteins and similar molecules involved in the immune response based upon genetic analyses. However, we cannot rule out the possibility that the ZP protein domains mimic those utilized for recognition purposes in the immune system without sharing primary sequence homology. Such similarities in structure can only be determined by performing protein crystallization studies.

The oligosaccharides coating the mZP glycoproteins have also been investigated, primarily because they have been implicated in the initial binding interaction. In 1985, Wassarman and colleagues proposed that O-glycans mediate the initial binding of murine sperm to its homologous eggs.[1] The primary carbohydrate motifs initially implicated in this process included terminal Galα1-3Gal and β-linked GlcNAc sequences.[11,12] However, mice lacking functional α1-3 galactosyltransferase retain their fertility and sperm binding activity.[13,14] Sperm from transgenic mice that are deleted in a specific sperm surface β-galactosyltransferase that acts as the putative receptor for the terminal GlcNAc sequence also retain their fertility and have enhanced sperm binding activity.[15] In addition, O-glycans terminated with GlcNAc are not prevalently expressed on the mZP.[16,17]

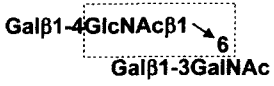
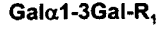
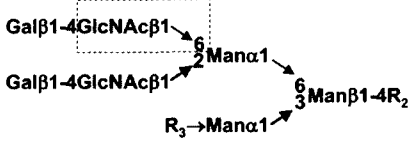
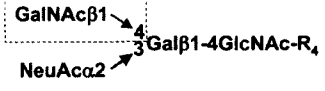
A more recent model based on previous inhibition studies, structural analyses of the mZP glycans, and substantial carbohydrate dependent binding of murine sperm in a somatic cell adhesion model suggests that β1-6 linked N-acetyllactosamine sequences linked to N- and O-glycans participate in the initial binding interaction.[16-18] Investigators in a recent study inactivated a gene essential for a glycosyltransferase (Mgat1) that is obligatory for complex N-glycan sequences in mouse oocytes by employing the Cre recombinase system.[19] Though eggs for these mice specifically lacked N-glycans bearing β1-6 linked N-acetyllactosamine sequences, they retained their fertility, indicating to these investigators that such sequences are not obligatory for sperm binding. However, since β1-6 linked N-acetyllactosamine sequences are also expressed on core 2 O-glycans in the mZP, murine sperm may also be able to bind via these sequences.[16,17]

Murine sperm undergo the acrosome reaction following their initial binding to the egg.[20] This process leads to the fusion of the plasma membrane with the outer acrosomal membrane followed by the blebbing off of the fused membranes to expose the inner acrosomal membrane.[21] The sperm then undergo a secondary binding interaction to the egg via this newly exposed membrane. A monoclonal antibody (Tec-02) directed against the GalNAcβ1-4Gal sequence blocks the secondary but not the initial binding of sperm to the mZP.[22] GalNAcβ1-4Gal sequences are primarily presented in the context of the Sd^a antigen on the mZP (Table 1).[16,17] Therefore both the primary and secondary binding interactions are likely mediated by carbohydrate sequences associated with the mZP.

The carbohydrate sequences implicated in these binding events are also upregulated in different immune cell populations during their activation (Table 1). This increased expression in immune cells is the direct consequence of the induction of several key glycosyltransferases necessary to synthesize these sequences. This linkage suggests that the immune and reproductive systems could be employing exactly the same carbohydrate sequences to mediate specific cell-cell recognition events. This type of vertical integration would make considerable physiological sense, based on consideration of each system's strategic imperatives.

The immune system can be broadly divided into two arms involving the innate and adaptive immune response.[23] The innate immune response is actually the more active arm, and in many instances recognizes carbohydrate sequences or peptidoglycans expressed on pathogens. This response is normally shared among all members of a species, and therefore should be more appropriately viewed as a system that targets "nonspecies" organisms for destruction (rather than the recognition of "nonself" in the classical immunololgical context). By contrast, the adaptive immune response is usually concerned with directing responses against peptide antigens.[24] It employs polymorphic major histocompatibility (MHC) molecules to mediate interactions between antigen presenting cells (usually dendritic cells) and lymphocytes, generating

Table 1. Murine immune activation ligands expressed on mZP glycoproteins

Carbohydrate Sequence*†	Activated Immune Cell Type	Induced Glycosyltransferase
	$CD4^+$ memory T cells	β1-6 N-Acetylglucosaminyltransferase (core 2 enzyme)[79]
	$CD4^+$ T cells	α1-3 Galactosyltransferase[80]
	$CD4^+$ memory T cells	β1-6 N-Acetylglucosaminyltransferase (GnT V or Mgat5)[81]
	Cytotoxic T lymphocytes	β1-4 N-Acetylglucosaminyltransferase (Sd^a enzyme)[82]

*Key to abbreviations for sugars: Fuc = fucose; Gal = galactose; GlcNAc = N-acetylhlucosamine; Man = mannose, NeuAc = N-acetylneuraminic acid (N-glycolylneuraminic acid [NeuGc] can substitute for NeuAc). † The key sequence added by the glycosyltransferase is indicated in the dashed box. R_1 = different N- and O-glycan sequence; R_2 = GlcNAcβ1-4GlcNAc-Asn or GlcNAcβ1-4[Fucα1-6]GlcNAc-Asn; R_3 = other antennae of complex N-glycans; R_4 = O-glycan core.

potent humoral and cell mediated responses directed against new peptide antigens. Because we have the experience of transplanting organs and observing their rejection as a consequence of mismatching MHC markers, we refer to this latter system as mediating the recognition of "self".[25]

By contrast, the reproductive system must subvert this recognition of "self" and promote the recognition of "species". Several differences are immediately apparent. First, the priority cells of the eutherian reproductive system (sperm and eggs) lack MHC molecules that define "self".[26-28] This absence is completely appropriate, since these cells are involved in recognition of their species complement but not "self". Secondly, instead of protein-protein recognition, the initial sperm-egg binding event in all species appears to be dependent upon a lectin-carbohydrate interaction.[29] *Thus it is possible that the carbohydrate sequences that are expressed on both the ZP and activated immune cells are being employed in both circumstances for immune recognition.*

This result suggests that there likely exists a carbohydrate dependent immune recognition system that is independent of mechanisms for the recognition of "self". This system basically is employed to define members of a species, as opposed to "nonspecies". We have previously referred to this alternate recognition system as the "species recognition system" (SRS).[3]

Where did the SRS originate? In the beginning, individual cells began to organize themselves into primitive multicellular colonies. It is likely that these colonies employed sticky protein-carbohydrate interactions to associate with each other. However, as the colony became more successful and the cells within it began to specialize, invading organisms likely became problematic. To deal with this problem, rudimentary immune cells developed that basically recognized carbohydrate sequences used to bind the colony cells together. This process enabled them to recognize cells of their own species and target the "nonspecies" invaders for destruction. Today we refer to this latter arm as the "innate immune response". Thus the SRS can be thought of as the "flip side" of the innate immune response. And like the innate immune response, the SRS also employs lectin-carbohydrate recognition to define cells of the same "species".

When sexual reproduction was initiated, it was only logical that the SRS carbohydrate sequences and their cognate receptors would be incorporated into the gametes so that they could be globally recognized by immune cells from all members of the same species. One additional benefit is that gametes could employ the preexisting SRS receptor-ligand systems to mediate primary and secondary binding events necessary for fertilization. A great economy was therefore realized by this vertical integration. This extension would also explain why glycosylation has been a very well conserved post-translational modification in eukaryotes: it is also employed for immune recognition processes.

This vertical integration served the vital imperatives of both the immune and emerging reproductive systems. It also likely greatly facilitated sexual reproduction, since alternative systems for immune recognition of gametes and for fertilization were unnecessary. And just as the immune cells could recognize other somatic cells of their own "species" within an organism, sperm released into an aqueous environment could also specifically recognize their homologous eggs even in the presence of eggs from many unrelated species by employing the SRS.

The Extension of Protection to the Developing Eutherian: Eu-FEDS

The SRS likely served the early sexually reproducing organisms well. The fact that sexual reproduction is now preferred form of propagation in all higher organisms is ample proof. There is evidence that there are systems for the recognition of "self" that actually predate MHC recognition.[30] However, the development of MHC recognition enabled an organism to evoke adaptive immune responses to more accurately target rapidly evolving pathogens, and therefore conferred a great advantage on organisms that acquired this system.

When the MHC system of recognition began to develop, it was simply integrated into the SRS. Though we do not have evidence (at least not yet), it is very likely that all tissues and cells express some SRS signals along with MHC molecules to facilitate the recognition of both "species" and "self" simultaneously. MHC recognition processes predominate in most tissues,

Table 2. Corollaries of eu-FEDS hypothesis

- The developing eutherian is protected by a system of soluble and cell surface-associated glycoconjugates that block immune and inflammatory interactions in utero in an MHC-independent manner. These glycoconjugates utilize their carbohydrate sequences as "functional groups" that enable them to mediate these biological activities.
- Mimicry or acquisition of such carbohydrate functional groups that are used in this protective system by pathogens or tumor cells enables them to either subvert or misdirect the eutherian immune response, thereby greatly increasing their pathogenicity.
- Expression of glycoconjugates used in this system by normal cells and tissues outside of the reproductive system protects them from immune responses, especially in those cases where MHC recognition is either absent or minimal.
- Another major recognition strategy now designated as the SRS coexists with MHC recognition in the immune system. The SRS functions to mediate recognition of "species" rather than "self". The SRS is utilized during gamete binding, the induction of the tolerant state necessary for reproduction, pathogenic subterfuge, and non-MHC-dependent recognition processes outlined in the first three corollaries.

but SRS takes the lead during reproduction and in those tissues where MHC expression is either weak or negligible. Therefore it is our working hypothesis that "immune privileged" tissues likely employ the SRS signals rather than MHC system to mediate immune recognition.

As noted previously, the innate immune response would actually handle the majority of the immune responses directed against pathogens. The adaptive immune response involving MHC recognition system would be directed against those pathogens that could not be adequately engaged by the innate immune responses. The SRS would enable reproduction and play a role in the immune recognition of cells, especially in the absence of MHC recognition.

For organisms that undergo external fertilization, protection within an organism had to be extended only to the gametes. For species undergoing internal fertilization, both male and female gametes plus the fertilized egg had to be protected. In the case of marsupial species, the system protecting progeny would only have to be operational for extremely brief periods of time. However, eutherians had to extend this coverage to their offspring for an extremely long period of time. In addition, they also had to circumvent the major problems associated with histoincompatibility. Therefore it is evident that eutherians employ a considerably more sophisticated system to protect their offspring. We refer to this system as eu-FEDS as noted earlier.[3] The primary corollaries for this hypothetical model are outlined in Table 2.

As noted earlier, the eu-FEDS model implies that glycoconjugates expressed in a specific temporospatial manner in the pregnant uterus suppress the human immune response directed against the fetus. In this model, the carbohydrate sequences linked to these glycoconjugates act as "functional groups" that allow them to mediate their immunomodulatory activities. These glycoconjugates work by disrupting essential immune cell adhesion events mediated by surface lectins and likely induce phenotypic changes in the immune effector cells. They could also interact with lectins that are coupled to specific signal transduction pathways that mediate inhibition of specific immune cell responses.

Another key concept in this model is that carbohydrate recognition is essential *but* not always sufficient for its induction.[3] In some cases, the protein carriers complement the carbohydrate binding interaction to confer specificity. Thus glycoproteins carrying different protein backbones but identical carbohydrate sequences could manifest completely different biological activities. Glycoproteins with identical protein structures but completely different carbohydrate sequences could also mediate completely different biological activities. However, in some instances the protein backbone is acting as a "platform" to present the appropriate carbohydrate functional group that actually mediates the activity.[3]

The major pregnancy associated glycoproteins implicated in eu-FEDS are shown in Table 3. Glycodelin-A is a major decidual product, accounting for 4-10% of the total glycoprotein made in this cell type. Expression of the fucosylated lacdiNAc sequence (GalNAcβ1-4(Fucα1-3)GlcNAc) sequence is relatively rare. The biantennary bisecting type N-glycan sequence associated with AFP is prominently expressed on many cell surfaces, but is virtually never found on circulating glycoproteins except during early fetal development and tumorigenesis. The sequence is also the most prevalent N-glycan expressed on CA125 derived from the ovarian tumor cell line OVCAR-3.[31] It is very likely that this sequence is also expressed on CA125 when it is synthesized in the pregnant uterus.[32] Many human mucins expressed in the human uterus express Le^x, Le^y and sialyl $Lewis^x$ sequences that may also modulate immune responses.[33,34]

Uromodulin is the major glycoprotein found in human pregnancy urine.[35] This component is a mixture of specific pregnancy specific glycoforms of Tamm-Horsfall glycoprotein (THP), the most abundant glycoprotein in this excretion.[36,37] However, THP from males and nonpregnant females is virtually inactive when tested in in vitro immune assays, whereas uromodulin is highly effective in the immune assays cited in Table 3.[38] Unlike THP produced in nonpregnant individuals, uromodulin expresses extremely unusual di- and trivalent sialyl $Lewis^x$ sequences.[39] Thus it is evident that many different glycoconjugates with immunomodulatory activities are expressed in the pregnant human female.

Eu-FEDS: The Strong Linkage to Pathogenesis

It is apparent that this system of immune modulation can also be linked to pathogenesis. The success of any pathogen is dependent upon its ability to survive the host's immune challenge for a sufficient amount of time to enable infection of another host. Therefore successful pathogens would be those that are persistent and couple themselves to the reproductive imperative, at least in part. In addition, such pathogens would also not interfere with their host's reproductive capability, so as to insure the availability of host organisms to continue the pathogen's existence.

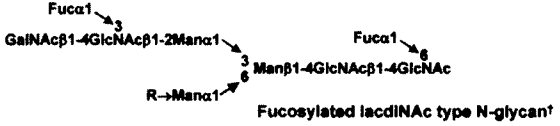
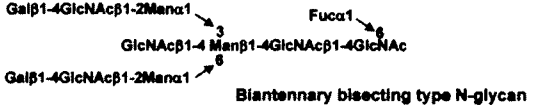
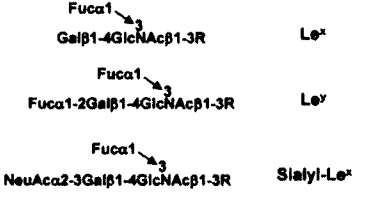
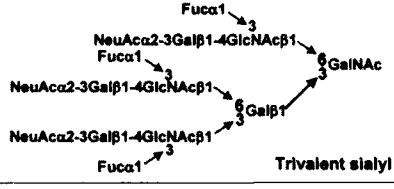
On the other hand, the most successful pathogens would be able to so highly integrate themselves itself into this system for reproductive protection that they would become virtually *invisible* to the host's immune system. Thus immune responses directed against them would mirror those against the developing eutherian, without adversely affecting the natural lifespan or reproductive potential of their host.[40] We believe that the degree of subterfuge would likely be commensurate with the pathogen's ability to either mimic or acquire key components involved in the protection of the gametes and the resulting progeny from immune challenge.

Mimicry or Acquisition?

How can investigators detect the "fingerprints" of either mimicry or acquisition of the system for reproductive protection by a pathogen? The obvious first choice is to determine if there are any overlapping gene sequences between the host and its pathogen unrelated to basic housekeeping functions (i.e., basic metabolic/catabolic pathways). However, there are several problems with this approach. First, molecular mimicry at the protein structural level may be achieved with little and perhaps no actual protein sequence homology.[41,42] Secondly, the pathogen may also use completely different molecules than the host to achieve the same effect i.e., functional mimicry. Finally, if post-translational modifications of key components represent the primary determinants associated with the protective effect, they would very likely not be uncovered by simple genetic screening.

A free-living pathogen would have to mimic key components of this system for reproductive protection using their own genes, gene products and post-translational machinery. By contrast, viruses could acquire specific post-translational modifications and in some cases the genes obtained from their host. Thus it would be far easier for viruses to obtain what they need from their host to integrate them into this system of reproductive protection. These capabilities may explain the extraordinary success of viral pathogens.

Table 3. Pregnancy associated glycoproteins expressed in the human

Pregnancy Associated Glycoprotein	Demonstrated Immune Modulatory Effects	Major Carbohydrate Sequence*	Pathogens Expressing Major Carbohydrate Sequence
Glycodelin-A	- Suppresses B cell mediated responses[83] - Downregulates T cell activation[84] - Induces apoptosis of activated T cells[85] - Suppresses NK cell cytolytic responses[86] - Inhibits E-selectin mediated adhesions[87] - Inhibits IL-2 synthesis[88]		Schistosomes[91]
α-Fetoprotein, CA125	- Suppresses NK cell mediated responses[76,89]		HIV-1 (gp120)[43]
Other Endometrial Mucins	Unknown at present		*Helicobacter pylori*[92,93] (Le^x, Le^y) HIV-1 Infected T lymphocytes[94] (Le^y only)
Uromodulin	- Binds IL-1, IL-2, tumor necrosis factor (TNF)[38] - Potently inhibits antigen driven T cell activation[38] - Potential E- and P-selectin ligand[90]		None known at the present time

*Abbreviations for sugars are identical to Table 1. R = different sugar sequences/carriers. † This sequence is also associated with human embryonal kidney 293 cell line.[95]

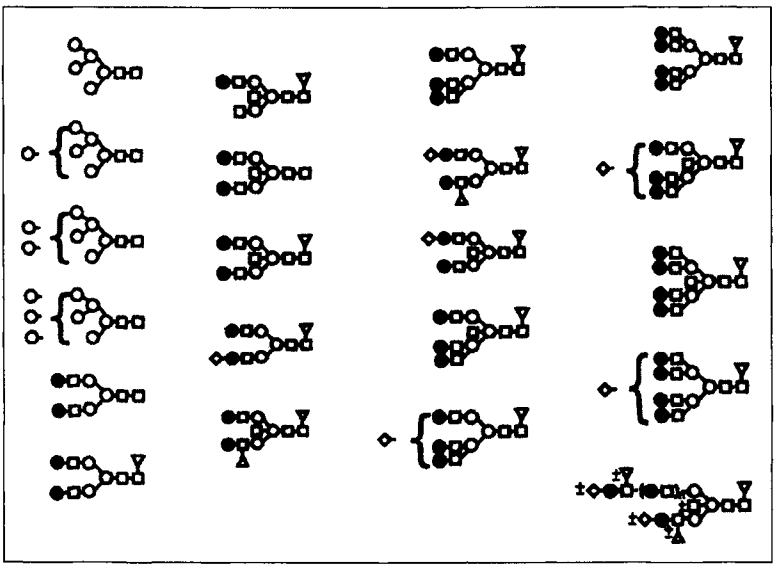


Figure 1. Profile of N-glycans associated with CA125. This figure is adapted from a study of all the carbohydrate sequences associated with this tumor mucin.[31] This profile is remarkably similar to the N-glycans linked to gp120 derived from Hiv-1 infected H9 lymphoblastoid cells.[43]

The key, of course, is to first understand the system of reproductive protection. There are likely many different overlapping systems that could provide protection in eutherians other than the current model discussed in this review. However, pathologists need to link them together into a sensible paradigm that can be employed to detect how pathogenic subterfuge could occur.

There is certainly inferential evidence that this subterfuge is occurring. For example, a compelling linkage is that helminthic parasites and bacteria are either mimicking or acquiring carbohydrate sequences implicated in immunomodulation (Table 3). Another example is that the profile of the N-glycans attached to CA125 expressed in OVCAR-3 cells is almost identical to those linked to gp120 expressed in HIV-1 infected H9 T lymphoblastoid cell line[31,43] (Fig. 1). Thus we believe that many different persistent human pathogens are utilizing these carbohydrate sequences as "functional groups" to integrate themselves into this system of fetoembryonic protection, thereby allowing them to couple their survival to that of the developing human.

AIDS: A Glycobiological Disease Linked to Eu-FEDS?

AIDS is caused by infection with the lymphotrophic lentivirus designated HIV-1.[44-46] Early symptoms closely resemble those associated with the common cold or flu viruses.[47] However, a complex array of symptoms usually manifests itself over an extended period of time, consistent with a gradual diminution in immune function eventually leading to death.[47] HIV-1 is the most thoroughly investigated virus in history.[48,49] However, in spite of this rather exceptional effort, many questions remain about how HIV-1 mediates its deleterious effects in the human immune system. This lack of understanding is particularly troubling for AIDS investigators, especially since all HIV-1 vaccines have been completely ineffective.[50,51]

In the early 1990s, several investigative groups proposed that live attenuated HIV-1 lacking essential gene products like Vpu or Nef could represent the best vaccines for AIDS.[52] However, because of the inherent danger in administering live attenuated HIV-1 variants to humans, this hypothesis was first tested in the SIV/macaque model system.

Macaques vaccinated with SIVmacC8, an SIV strain with a 12-base pair in-frame deletion in Nef [53] had unexpected rises in viral loads followed by disease progression.[54] Analysis of the

vaccine strain revealed restoration of the Nef deletion; the Nef gene gradually evolved to resemble the amino acid sequence of the parental virus clone (SIVmacJ5).[54] This reversion was also observed independently by other groups.

This recovery of the virus led to the construction of more highly attenuated strains of SIV. SIVmac239-[DELTA]3 is a Nef-deleted vaccine strain with additional large deletions in Vpu, the long terminal repeat that overlaps with the remaining Nef coding region, and the negative regulatory element.[55] However, inoculation with this strain led to the development of AIDS-like symptoms in 6/8 neonates.[55] A striking association between disease progression and the emergence of shorter viral genomes in the more effective SIV subtypes was also noted.[55,56] Adult macaques inoculated with SIVmac-239[DELTA]3 initially developed responses against this attenuated virus without overt signs of an AIDS-like condition.[55] However, a more recent report indicates that these monkeys are now unfortunately also contracting simian AIDS.[57]

Thus attenuated SIV variants with even more highly restricted genomes can overcome the macaque's immune response and evolve to become just as potent as the parental virus. This result was extremely surprising for virologists. The most relevant question that arises is *how could SIV isoforms with even more highly restricted genomes defeat the macaque's immune response?* The implication is that the surface glycoproteins are able to induce pathology. A plausible hypothesis based on our studies is that differential glycosylation of the SIV glycoproteins associated with these attenuated SIV variants generates different glycoforms capable of either inhibiting or aberrantly activating different immune cell functions.[2,3,40] By extrapolation, we also consider it to be extremely likely that HIV-1 could also completely dispense with its Vpu and Nef genes, yet still induce AIDS via an identical mechanism of action.

To understand how lentiviruses like SIV and HIV-1 are well equipped to utilize glycosylation to generate functional and structural diversity, it is necessary to understand some aspects of glycosylation.[2,3] Surface glycoproteins like gp120 represent supramolecular substrates for the glycosylation modification enzymes in the Golgi apparatus.[3] Therefore differential glycosylation of glycoproteins can be evoked by subtly mutating the protein sequence, thus enabling the resulting isoforms to interact with other Golgi enzymes. The mutant forms of lentiviral glycoproteins could thus acquire new carbohydrate sequences that were originally not accessible to the wild type viral glycoproteins.

This acquisition would enable the mutant glycoforms to access new functional capabilities compared to the wild type glycoproteins. The factors that contribute to structural and functional diversity of HIV-1 glycoproteins are summarized in Figure 2. Thus all of these factors working together create an enormous diversity of glycoforms, each with a potentially different biological activity. Because the number of virions that actually initiate infection is relatively low, this diversity can only be achieved after many generations of virions are created within the host. Therefore this process is "evolutionary", requiring considerable time for the induction of pathological responses in the host. This result is consistent with the time course of HIV-1 infection and the long period of relative latency providing ample time for transmission to another host.

It is therefore easy to see that by acquisition of specific carbohydrate functional groups associated with eu-FEDS, lentiviral glycoproteins could also induce immune suppression in infected individuals. However, if these functional groups were presented in an inappropriate context to immune effector cells, they may also be able to evoke abnormal activation of different immune responses. *Thus differential glycosylation could explain another central paradox observed in AIDS: the coexistence of both immune suppression and activation processes.*

Are there any direct linkages between HIV-1 and the pregnant human uterus? It is apparent that gp120 HIV-1 expresses the bisecting biantennary type N-glycan implicated in the suppression of NK cell mediated responses (Table 3). We have already highlighted the overlap in glycosylation between CA125 and gp120 in the previous section. It is very possible that HIV glycoproteins could acquire many different carbohydrate sequences in different cell types. Studies are currently underway to test for other linkages that tie eu-FEDS to the development of AIDS pathogenesis.

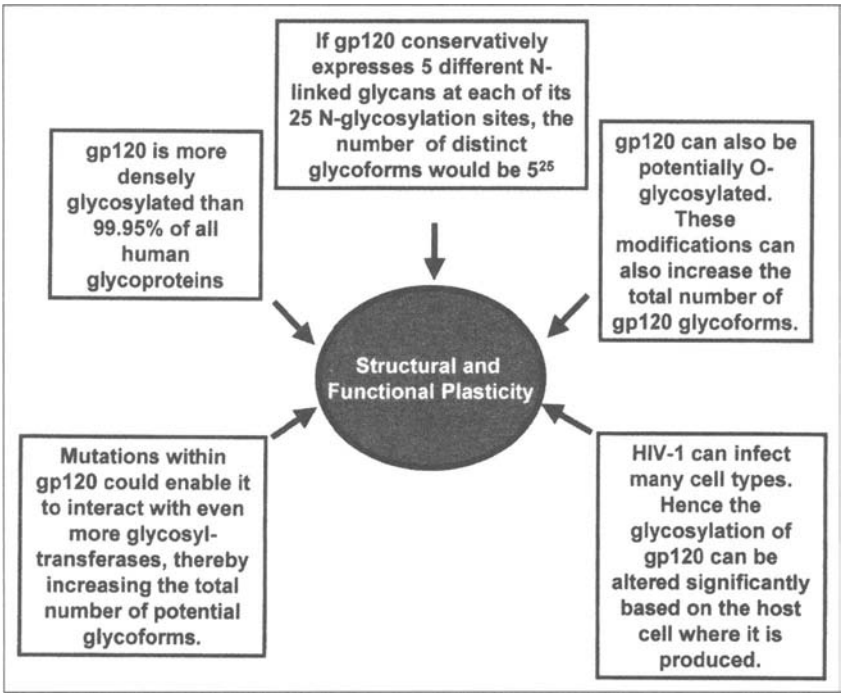


Figure 2. Glycosylation factors that contribute to functional diversity of HIV glycoproteins.

SIV Infection of Its Natural Hosts: The "Perfect Eu-FEDS Pathogen"?

Another very significant immunological enigma associated with lentiviral infections is their ability to induce pathology in heterologous but not their natural hosts. For example, SIV infection of African green monkeys (AGMs) and sooty mangabees (SMs) does not lead to the development of simian AIDS in these natural hosts. However, infection of heterologous hosts like rhesus monkeys and cynomolgus macaques with exactly the same viral isolates leads to the development of simian AIDS.[58]

SIV infection results in the induction of a profound viremia in both the natural and heterologous hosts.[59,60] In the heterologous hosts, the viral load drops to a moderate level, but increases with disease progression. However, in AGMs and SMs, the plasma viral load remains high without the inducing pathology during the entire lifetime of the monkeys.[61,62] SIV infection of macaques and rhesus monkeys leads to the generation of neutralizing antibodies, anti-Gag antibodies, and potent cytotoxic T lymphocyte responses directed against the virus.[58] By contrast, similar infection of its natural hosts leads to little if any humoral or cell mediated responses directed against SIV. Therefore a very potent response to SIV is correlated with the development of simian AIDS, whereas nearly a complete lack of response in its natural hosts leads to no pathological effect. The ability of SIV to induce tolerance to both humoral and cell mediated responses in its natural hosts is quite remarkable and not easily explained by employing classical immunological paradigms.

We have pointed out in a previous review that the very mild response to SIV in its natural hosts is almost exactly like that of the mother to her histoincompatible fetus.[40,63] Therefore it is possible that unlike its heterologous hosts, the glycosylation system of the AGMs and SMs attach the appropriate SRS carbohydrate signals in the right context to their glycoproteins, thereby preventing the development of adaptive immune responses directed against this virus.

Testing of this hypothesis will require the elucidation of the SRS signals in these animal models and the subsequent demonstration that such signals are uniformly acquired by SIV in diverse immune cell types in its natural but not heterologous hosts.

Cancer and the Protection of the Developing Eutherian

Another significant pathogenic process in many organisms is the development of malignant tumors. In humans, there has been evidence suggesting for some time that the escape of tumor cells from the immune response could be linked to the maternal-fetal system of protection.[64,65] For example, many glycoproteins associated with the pregnant uterus are also secreted by tumor cells. These include α-fetoprotein (AFP),[66] carcinoembryonic antigen (CEA),[67,68] CA19-9 antigen,[69] and CA125.[70,71] Initially, such components were detected in the uterus, the developing human or on malignant tissues using either monoclonal or polyclonal antibodies. Therefore they are often referred to as "oncofetal antigens".

The problem with many of these previous studies is that many factors have not been sufficiently purified for in vitro immune assays. For example, a clinical test for CA125 has been employed for some time to diagnose the recurrence of ovarian cancer.[72] However, it was not until quite recently that the gene for CA125 was cloned[73-75] enabling its purification and subsequent elucidation of its glycosylation profile.[31] This analysis in turn led to studies that revealed its ability to suppress natural killer (NK) cell cytotoxicity.[76] This effect may be crucial for tumor development. It is well established that tumor cells lose their MHC class I expression during oncogenic progression.[77] This loss should make them more highly resistant to cytotoxic T lymphocytes but more sensitive to NK cells.[77] The secretion of mucins like CA125 may enable the tumor cells to escape NK cells, thus making them immune to virtually all cell mediated responses.[76,77]

Another problem is that the post-translational modifications of glycoproteins could change during tumorigenesis, just as they do during pregnancy. A good example is the shift in the glycosylation of α-fetoprotein expressed in the amniotic fluid of gravid human females.[78] Another complication is that these proteins and mucins are often cloned and expressed in cell types that do not recapitulate the natural glycosylation state found in the tumors. This lack of appreciation for the functional role of glycosylation could in turn lead to the false impression that they have no immunomodulatory activity. Therefore as more consideration is given to the role of glycosylation in post-translational modifications of such factors, the more evidence will likely be obtained that supports this linkage. However, even now it is evident that malignant tumors in all species likely couple their survival to the reproductive imperative inherent in the eu-FEDS and SRS paradigms.

The Future

The concept that glycoconjugates could play a major role in mediating accommodation of the human fetus was first presented in 1996.[1] The linkage of this protective effect to AIDS and other persistent pathogenic states including cancer was first elaborated the following year.[2] The model was further updated in 2001 to include the definition of the SRS and the extension of this system to all eutherian species.[3] Today although there are still many major technical problems with securing absolute proof for this hypothetical model, we are more confident in this paradigm than ever.

One very major limitation to performing these studies is access to key glycobiological reagents that could enable investigators to address these issues experimentally. When the international Consortium for Functional Glycomics funded by the National Institutes of Health (NIH) was first initiated in 2001, one of its major goals was to provide scarce glycobiological reagents to investigators working in many different areas. However, because many of the clones of essential glycosyltransferases are currently under patent protection, it is impossible for investigators to obtain the key enzymes they need to test this hypothesis in depth. An apt analogy in this circumstance would be to require molecular biologists to perform DNA sequencing without

access to either endonucleases or taq polymerases. Therefore progress in this area has certainly been impeded by the lack of access to these reagents.

Another difficulty is the political problems encountered when trying to promote a novel concept such as eu-FEDS in the context of other competing paradigms. However, a detailed discussion of these problems will be more appropriate in another future forum.

A way of overcoming these obstacles may be the emerging interest in the eu-FEDS model within the AIDS community. Certainly, glycobiological subterfuge mediated by HIV-1 glycoproteins will very likely be the key to finally understanding AIDS pathogenesis, as we have clearly indicated in this review. AIDS investigators and their contacts in the lay community have literally worked miracles to overcome political and logistical impediments. This same interest is now being directed to assist studies that develop the eu-FEDS hypothesis. If we can go forward with these studies, then they could potentially benefit not only AIDS patients, but also innumerable individuals suffering from many different persistent pathological states that we have outlined here.

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CHAPTER 18

The Nature and Role of the Decidual T Cells

Lucia Mincheva-Nilsson and Vladimir Baranov

Abstract

The immunological paradox of mammalian pregnancy is the acceptance of the fetus, a semiallogeneic allograft that normally should provoke an immune response of the maternal T cells leading to fetal rejection. In this chapter the current understanding of decidual T-cell immunobiology is reviewed and discussed. Previous and current results about decidual γδT-, αβT-, NKT- and regulatory-T cells are summarized. The significance of thymic involution in pregnancy and extrathymic T-cell differentiation in decidua as mechanisms creating maternal tolerance towards the fetus is considered. Available evidence regarding T-cell contribution to the immune tolerance is integrated into a hypothetical model. An outline of recent findings supporting the increasing role for γδT-, NKT- and innate regulatory T cells in the maintenance of pregnancy is given. We conclude that decidual T cells play a central role in pregnancy and a greater knowledge of their immunobiology is of crucial importance in the understanding of fetal tolerance.

Introduction

The immunological challenge of mammalian pregnancy is to exert immunosuppression of specific responses towards the fetus without compromising the ability to fight infections. Pregnancy creates a physiological state of immunotolerance towards the fetus. The molecular mechanisms responsible for this tolerance are still poorly understood. Clearly, no single mechanism can completely explain fetal tolerance. Instead a jigsaw puzzle of cell types, cytokines, enzymes and hormones is operating at the maternal-fetal interface molding the frame of a successful pregnancy. Previously, it was hypothesized that the pregnancy is "hidden" from the maternal immune system since the trophoblast does not express classical antigen-presenting molecules of the major histocompatibility complex (MHC). It is now a generally accepted knowledge that the maternal adaptive immune system recognizes fetal antigens, as judged by alloantigen-specific alterations in the maternal T- and B-cell phenotypes during pregnancy.[1-4] The pregnancy is recognized in such a way that the specific, acquired arm of the maternal immunity is suppressed and the innate, non-specific, "first line of defense" arm is used and promoted to compensate for the loss of specific immune functions.[5,6] In this chapter we will focus exclusively on the decidual T cells, their immunobiology and a possible contribution to the immunosuppressive state in decidua.

T Cells Are Constitutive Members of the Decidua-Associated Lymphoid Tissue (DALT)

The pregnant uterine mucosa, the so called decidua, is rich in immune cells, illustrated in Fig. 1 by immunoperoxidase staining for the common leukocyte antigen CD45. In human early pregnancy decidua about 15-30% of all cells are leukocytes.[7,8] The decidua associated

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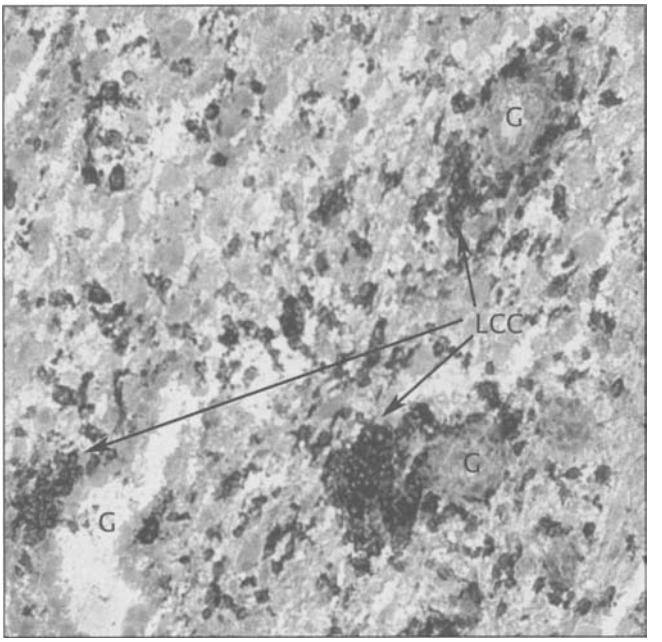


Figure 1. Immunohistochemical staining of immune cells in human early pregnancy decidua with monoclonal antibody against the leukocyte common antigen CD45 showing the histological organization of DALT. LCC = lymphoid cell clusters, G = decidual glands. Magnification, X 32.

lymphoid tissue (DALT) comprises mainly of $CD56^{bright+}/CD16^{-}$ NK-like cells, T cells bearing T-cell receptor (TCR) αβ or γδ, dendritic cells and macrophages. B cells are scarce or absent. There is a distinct histological organization of DALT into: (1) aggregates of cells, named lymphoid cell clusters (LCC), found close to endometrial glands and blood vessels (Figs. 1 and 2); (2) subepithelial lymphoid cells contacting the basolateral part of the epithelium of the decidual glands; and (3) individual cells randomly dispersed between the decidual stromal cells[7,9,10] (Fig. 1). The LCC seem to be the nearest human analogue of the cryptopatches in the murine intestine.[11] The LCC have a "follicle-like" structure but lack B cells. They are further composed of $CD56^{+bright}$ cells, T cells (Fig. 2) and mactophages/dendritic cells.[12] The majority of the cells in the LCC are activated and express a variety of phenotypic-, adhesion-, memory- and activation markers (CD2, CD3, CD4, CD7, CD8, CD45RO, CD94, CD38, CD69, CD71, HML-1) and MHC class II of all three loci (DR, DP and DQ) suggesting that the LCC are sites for activation, cooperation and differentiation of lymphoid cells.[7,10] In contrast to other mucosal sites there are no truly intraepithelial lymphocytes in decidua, i.e., immune cells located above the epithelial basal membrane of the decidual glands. However, numerous decidual lymphocytes, both $CD56^{+}$ and T cells, are in close contact with the basal part of the glandular epithelium.

Characterization of the Decidual T Cells According to TCR Usage and Phenotype

Classically, T cells are defined as lymphocytes that express clonally distributed T cell multi-chain receptor complex (TCR-CD3). The polypeptide chains of the TCR form the antigen and MHC binding unit which is non-covalently associated with the CD3 chains that transduce the signals inside the T cell. After antigen-dependent maturation the cell is able to elicit a variety of immune functions like cytokine production, cytotoxicity, suppression etc.

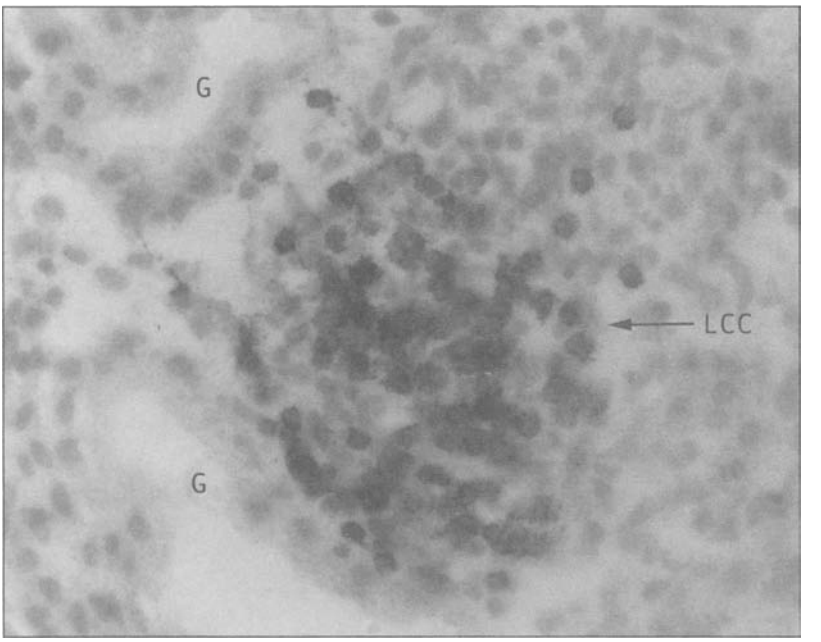


Figure 2. Immunohistochemical staining of decidual T cells in a lymphoid cell cluster (LCC) using a monoclonal antibody against CD3. Magnification, X 60.

Two lineages of T lymphocytes can be defined in higher vertebrates by the type of polypeptide chains expressed in the TCR – αβT cells expressing an α and a β polypeptide chain and γδT cells expressing a γ and a δ chain. The majority of T cells, developed in the thymus, express a TCRαβ. About 90 to 95% of circulating T cells are αβT cells while γδT cells constitute only a small proportion. A part of the γδT cells is thymically generated early in ontogeny, but a major fraction is generated in an extrathymic compartment.[13] In adult animals and humans γδT cells can be roughly divided into two groups – (i) Vγ9/Vδ2$^+$ circulating lymphocytes comprising 1-10% of the peripheral blood mononuclear cells and (ii) Vδ1$^+$ resident cells of the mucosal surfaces of the digestive-, respiratory-, and urogenital tracts, and murine skin. In some reports the γδT cells are as abundant as 50% of the T cells in the murine skin epithelia or gut mucosa.[14,15] Although classified as T cells by their TCR, the αβ- and γδT cells seem to be different in their immune biology and belong to separate branches of the immune system – the TCRγδ cells act like innate immune cells while the TCRαβ cells play a central role in the adaptive immune system.[16] As a background to the discussion of the decidual αβ- and γδT cells a summary of the major properties of the TCR αβ and γδ cells is given in Table 1.

The decidual T cells (Fig. 2), not as numerous as the CD56$^{+bright}$/CD16$^-$ cells, constitute the second largest lymphocyte subpopulation, accounting for 20-30% of the leukocytes in human first trimester decidua.[7,17,18] Both TCRαβ and TCRγδ cells are present in approximately equal proportions. The dominating population of lymphoid cells in human decidua – the CD56$^{+bright}$/CD16$^-$ cells, present in the endometrium already prior to pregnancy, rapidly and drastically drops in numbers as the pregnancy proceeds and is practically absent at term. The T-cell population, however, persists and is even relatively enriched throughout the pregnancy.[19] There have been conflicting reports regarding the T-cell population in decidua in human and animal studies. Others and we[17,20,21] have reported accumulation of activated γδT cells, while some[22-24] reported few or absent CD3$^+$ cells in decidua. This led several authors to conclude that T cells are unlikely to play a role in the maintenance of pregnancy.[25]

Table 1. Comparison between the γδT and αβT lymphocyte lineage

Characteristics	γδT Lymphocytes		αβT Lymphocytes
	Resident	**Circulating**	
Immunity affiliation	innate	innate/adaptive (?)	adaptive
Ontogeny	develop first	develop first	develop second
Development	thymus/extra-thymus	thymus	thymus/extra-thymus
Ag-receptor configuration	$CD3^+V\delta1$	$CD3^+V\delta2$	$CD3^+TCR\alpha\beta$
Phenotype	$CD4^-/CD8^-$ (most) $CD8^+$ or$CD8\alpha\alpha$ (some)	$CD4^-/CD8^-$ (most)	$CD4^+$ or $CD8^+$
MHC restriction	no	no	yes
Antigen recognition	self-antigens	foreign non-peptide antigens	peptide+MHC
Frequency in blood	very few	1-10%	65-75%
Tissue distribution	mucosae, epithelia, lymphoid tissues	blood lymphoid tissues (?)	blood, lymphoid tissues, mucosae
Effector functions	cytoxic potency cytokine release	cytotoxic potency cytokine release	cytotoxic potency cytokine release (Th1/Th2)
Biological functions	immunoregulation immunosurveillance pathogen eradication epithelium repair (mice)	pathogen eradication	pathogen eradication immune protection

This apparent discrepancy of results and misjudgment in the interpretation may be partly explained by different sensitivities of antibodies and techniques used and the significant downregulation of the TCR/CD3 complex on T cells in pregnant uteri of both humans and animals.[18,21,26,27] The $CD3^+$ cells in decidua differ phenotypically compared to these in peripheral blood. Several activation markers such as CD69, HLA-DR, -DP and -DQ and the memory marker CD45RO are expressed on decidual T cells from early human normal pregnancy.[28,29] Functionally, the human decidual T cells are able to respond to in vitro stimulation by lectins and mitogenic anti-CD3 antibodies, however their responses against alloantigens are selectively down-regulated as measured by suppressed in vitro proliferation in mixed lymphocyte culture reaction.[17,30] Below follows a separate description of the four important subsets of T cells in decidua – γδT cells, αβT cells, NKT cells and regulatory T cells.

Decidual γδT Cells

The γδT cells are present in endometrium of all mammals throughout pregnancy.[26] It has been shown that these cells specifically colonize the non-pregnant murine and sheep endometrium and show a dramatic increase during pregnancy suggesting a special role at the feto-maternal interface.[31] The number of γδT cells in the uterus is higher in allogeneic than syngeneic pregnancy, and the expression of the TCRγδ in the pregnant uterus is shown to be hormonally controlled.[32] The decidual γδT cells are large granular lymphocytes rich in intracytoplasmic granules and express neither CD4 nor CD8 (double negative).[7,17,33] They are $CD2^+$, express the activation marker CD69, the memory/activation marker CD45RO, the NK receptors CD94[17,33] and NKG2D, and CTLA 4 (Fig. 3). The human γδT cells (Fig. 4) comprise a heterogeneous population: double positive $TCR\gamma\delta^+/CD56^{+dim}$ cells and TCRγδ single positive cells (Fig. 5).[33] The counterpart of these cells in the murine system seems to be the $TCR\gamma\delta^+$/asialoGM^+ cells and the single positive murine γδT cells as described by Arck et al.[34] The

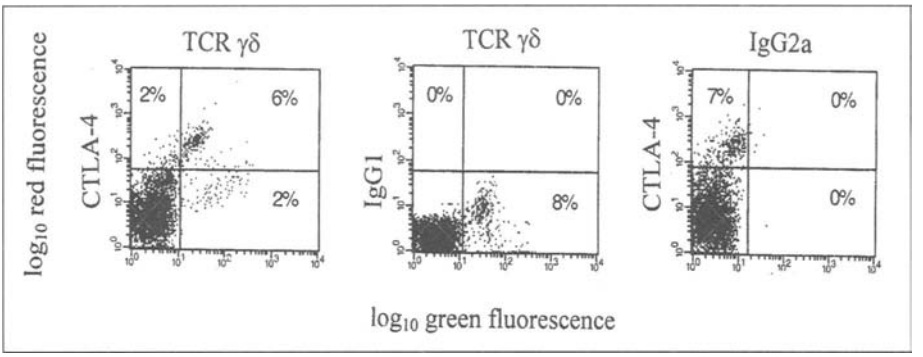


Figure 3. Flow cytometric analysis of the regulatory cells-associated marker CTLA4 expression on freshly isolated, non-permeabilized human decidual γδT cells from early normal pregnancy. Reprinted with permission from ref. 10.

surface density of the TCR/CD3 is low in freshly isolated decidual γδT cells[17,18,35] but can be up-regulated in vitro.[17]

The vast majority of the human decidual γδT cells is Vδ1$^+$.[33,36] Itohara et al[37] and Heyborne et al[38] have shown that the Vδ1 chain is also preferentially used by γδT cells in the uterus of

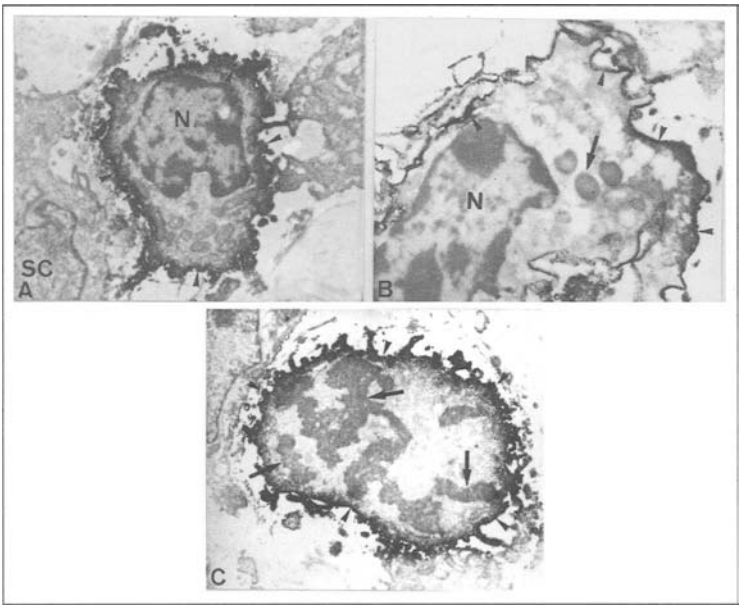


Figure 4. Immunoelectron microscopy of γδT lymphocytes in the first trimester decidua stained with anti-TCRγδ mAb using an indirect immunoperoxidase method. No additional counterstaining was performed. For detail information see L. Mincheva-Nilsson et al 1997. A) γδT lymphocyte showing an intense cell surface staining (arrowheads) and a close contact with stromal cell (SC). N= nucleus. Magnification, X 6,000. B) A portion of the γδT cell cytoplasm containing characteristic cytoplasmic granules (arrow). Arrowheads show the surface immunostaining. N= nucleus. Magnification, X 22,000. C) Electron micrograph of a dividing γδT cell. Arrows point to chromosomes. Note the positive staining of the cell surface staining (arrowheads). Magnification, X 7,000.

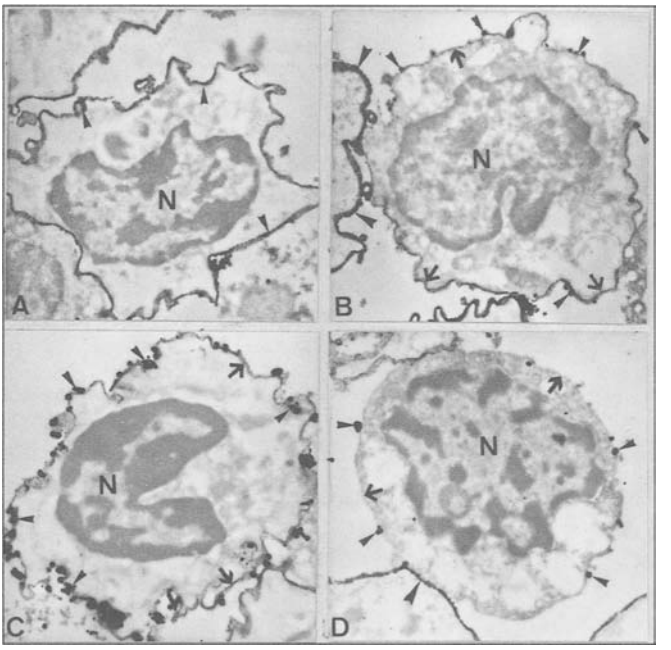


Figure 5. Immunoelectron micrographs showing four different cell surface phenotypes in isolated decidual mononuclear cells stained for colocalization of TCRγδ by the gold/silver method and for CD56 by the immunoperoxidase method as described in L. Mincheva-Nilsson et al 1997. No additional counterstaining was made. A) The plasma membrane of a $CD56^{+bright}$ cell intensively stained by the peroxidase reaction product (arrowheads). N= nucleus. Magnification, X 9,000. B) The plasma membrane of a $CD56^{+dim}$/$TCR\gamma\delta^{+low}$ cell exhibits weaker staining with the peroxidase reaction product (arrows) compared with the intense surface staining of an adjacent $CD56^{+bright}$ cell (thick arrowheads). A few gold-silver grains showing the expression of TCRγδ can also be seen on the plasma membrane (thin arrowheads). N= nucleus. Magnification, X 9,500. C) The cell surface membrane of a $CD56^{+dim}$/$TCR\gamma\delta^{+high}$ cell displays weak peroxidase staining (arrows) and numerous surface gold-silver grains (arrowheads). N= nucleus. Magnification, X 9,000. D) The plasma membrane of a $CD56^{-}$/$TCR\gamma\delta^{+low}$ cell is not stained by the peroxidase reaction product (arrows) but exhibits rare gold-silver grains (thin arrowheads). Note the intense surface staining of a neighboring $CD56^{+bright}$ cell with the peroxidase reaction product (thick arrowheads). N= nucleus. Magnification, X 9,500. Reprinted with permission from ref. 33.

non-pregnant and pregnant mice. Thus, the γδ T cells in pregnant uterine mucosa, like other mucosa- associated γδT cells, are resident $V\delta1^{+}$ cells.

Similar to resident γδT cells at other mucosal sites,[5,39] the decidual γδT cells are "activated but resting". We have shown that they possess a cytotoxic potency and express five major cytolytic molecules: perforin (Pf), granzyme A, granzyme B, granulysin and Fas ligand (FasL), and store them in microvesicles in intracytoplasmic cytolytic granules (Fig. 6).[40] Like other cytotoxic lymphocytes[41] the decidual γδT cells do not express FasL on their surface but store preformed FasL in the granules (Fig. 6), and can rapidly mobilize it to the cell surface upon stimulation.[40] Thus, the two major cytotoxic mechanisms—Pf- and FasL-mediated—are performed by one common secretory pathway based on cytolytic granule exocytosis.[40] Cytotoxic mechanisms play a crucial role in the clearance of viral and bacterial infections, tumor surveillance, transplant rejection, homeostatic regulation of immune responses and peripheral tolerance.[42] Logically these mechanisms should have an important function at the feto-maternal interface by protecting the maternal-fetal unit against pathogens, controlling invasion of placental trophoblast, and creating a local transient immunotolerance toward the semiallogeneic

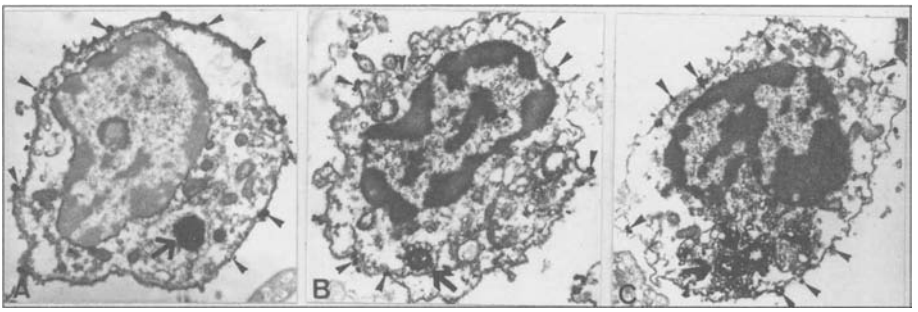


Figure 6. Immunoelectron micrographs of isolated decidual mononuclear cells stained for colocalization of TCRγδ by the gold/silver method and perforin, Fas ligand and granulysin by the immunoperoxidase method. For technical data see L.Mincheva-Nilsson et al 2000. A) The plasma membrane of a γδT cell is labeled by numerous gold/silver grains (arrowheads). The peroxidase reaction product stains a core of a cytolytic granule indicating the presence of perforin (arrow). Magnification, X 12,000. B) TCRγδ-positive cells showing surface immunostaining by gold/silver grains (arrowheads). Arrow indicates Fas ligand-positive cytolytic granule. Magnification, X 12,000. C) γδT cell displaying the cell surface gold/silver staining for TCR. Arrows indicate two cytolytic granules stained by the electron-dense peroxidase product showing the presence of granulysin. Magnification, X 12,000.

conceptus through deletion of fetus-reactive lymphocyte clones. Indeed, recent studies of Pf- and FasL-deficient mice have shown that although functional deletion of Pf or FasL alone does not appear to affect fertility, the combined absence of these two effector molecules induces infertility.[42]

Interestingly, we were able to stain γδT cells in mitosis (Fig. 4)[33] proving that the γδT cells divide in human decidua. As a rule, the plasma membrane of the mitotic cells was strongly stained with the reaction product indicating that a high level of TCR expression is necessary to enter the proliferative pool.[43] Our finding of γδT cells dividing in situ is in line with the previous suggestion that γδT cells might expand in epithelial sites exposed to external environmental antigens, and, in some cases, recognize self-antigens, specific to a particular local environment.[14,15,44] By analogy, decidual Vδ1$^+$ T cells may recognize trophoblast-related antigens and be involved in the control of trophoblast invasion during placenta formation.[45]

Hayakawa et al[46] and Kimura et al[47] have shown expression of mRNA for RAG-1 and RAG-2 proteins, which are required for TCR rearrangement, in human CD56^{bright}/CD16$^-$ cells and in murine decidual mononuclear cells respectively. We have confirmed and extended these results showing that transcripts of RAG can be easily detected in purified CD56$^+$, CD2$^+$, *c-kit*$^+$ or IL-7R$^+$ decidual cells implying an ongoing process of TCR gene rearrangement (Fig. 7).[33] There is no doubt that continuous rearrangement of TCRγδ takes place in decidua, probably for two purposes: (1) local extrathymic differentiation of γδT cells by TCR receptor rearrangement and (2) secondary TCRγδ rearrangement, permitting revision of antigen receptors on mature cells, thus adjusting the decidual γδT cell repertoire to the ongoing pregnancy. RAG re–expression, involved in receptor editing and revision, is a phenomenon observed in the lymphoid periphery[48] and seems to be required for shaping the T cell repertoire during development and peripheral tolerance.[49] Although not proven yet it is reasonable to assume that both local TCRγδ receptor rearrangement and revision are equally used in decidua.

Is there a need for extrathymic T-cell differentiation in decidua? What biological significance there may be for this phenomenon during pregnancy?

We can argue for at least two different reasons for extrathymic maturation in pregnancy. The first reason is priming the maternal immune system to the fetus. The meeting between the mother and the fetus is dual: (1) between the maternal blood and syncytiotrophoblast cells of the chorion villi of the placenta and (2) between the extravillous trophoblast and the maternal

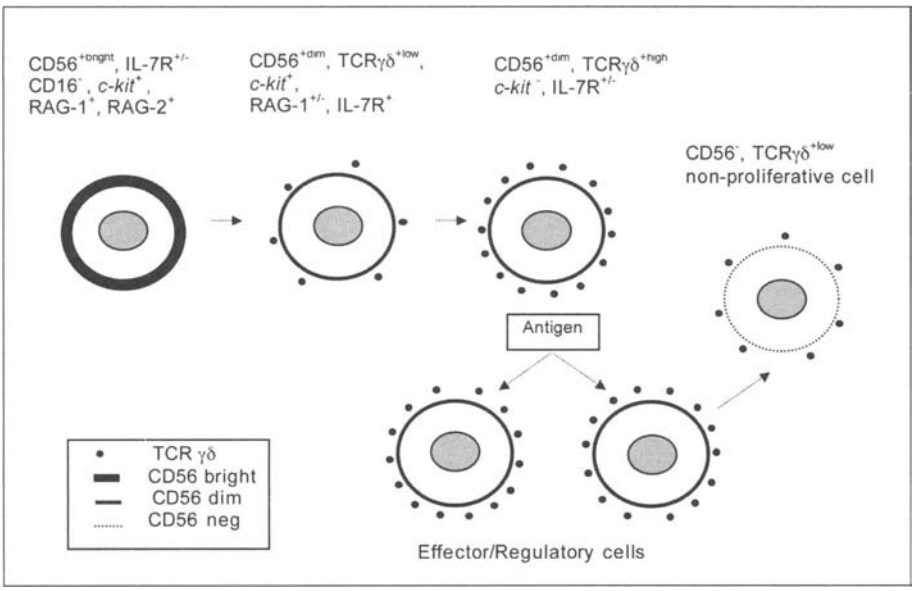
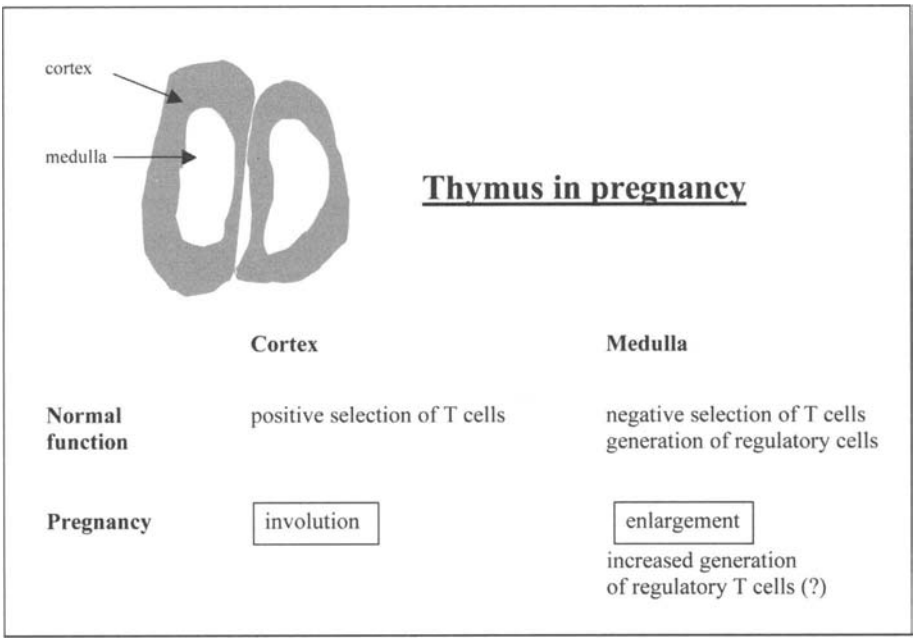


Figure 7. A suggested pathway of decidual γδT cell development. Under the influence of the decidual environment, a subset of *c-kit*$^+$, CD56$^{+bright}$ cells activates the RAG1 and RAG2 genes and starts the TCR gene rearrangement. If these cells express IL-7R, they receive a signal that results in a commitment to the γδT cell lineage. The heterogeneity of TCRγδ expression within CD56^{+dim} cells reflects successive steps of differentiation. The CD56^{+dim}/TCRγδ^{+high} cells represent a functionally mature subset. Dividing CD56^{+dim}/TCRγδ^{+high} cells reflect a direct primary antigen-driven proliferation of these cells. The CD56 expression is finally lost, and the cells down-regulate their TCR and thus become terminally differentiated cells. Reprinted with permission from ref. 33.

epithelial, stromal, endothelial and immune cells in decidua when placenta is formed. It is reasonable to assume that the first encounter and presentation of fetal antigens to the immune system takes place in decidua. We hypothesize that the CD56$^{+bright}$ cells harbor a population of progenitor cell of bone marrow origin. CD56, the neural-cell adhesion molecule,[50] binds to endothelial cells and subendothelial matrix and thus is used to enrich these cells to the decidual compartment. The cells will further differentiate/rearrange TCR locally (alternatively, naive thymus-derived T cells will revise their TCR) upon the encounter of fetal antigens. The extrathymic maturation in decidua might be one of the mechanisms adjusting the immune system and the T-cell repertoire towards acceptance of the ongoing pregnancy. Heyborne et al[45] have shown that murine decidual γδT cells recognize trophoblast-derived antigens. Immune cells, locally primed in decidua might then repopulate the peripheral blood of the pregnant woman as suggested by published reports, reviewed in reference 20.

The second reason for extrathymic maturation in decidua might be the transient thymic involution taking place during pregnancy in a number of species including rat, mouse, and humans. A great loss in thymic weight occurs due to a blockage in the cortex of T-cell development at the early CD44$^+$/CD25$^+$ pre-T cell stage. The pregnancy hormones estrogen and progesterone, and their receptors are regarded as the agents causing cortical thymocyte loss.[51,52] The cortical involution is at its greatest by midpregnancy and is maintained until lactation ceases. The implications of the observed thymic changes can be anticipated if they are correlated to the known thymic function (Table 2). Although the functional importance of pregnancy-induced thymic cortical involution remains unknown, some speculation is possible. It has been shown that T cells recognizing paternal antigens undergo clonal deletion or anergy.[3] If the goal of the

Table 2. Correlation between the thymic cortex and medulla and their function in normal situation and pregnancy



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immune system is to remove potentially harmful T cells from the peripheral circulation,[3] a logical adjunct to this goal would be to block the production of new T cells that could replace clonally-deleted paternally-reactive T cells. It is tempting to interpret the simultaneous enlargement of the thymic medulla in pregnancy[52] as a potential increase in production of regulatory T cells needed to modulate the maternal immune responses.[53] It is interesting to note that the decreased T cell output from the thymus does not induce significant quantitative changes in the peripheral population of T cells during pregnancy.[51] Presumably the long-lived populations of peripheral lymphocytes and/or extrathymic T cell differentiation prevent their drop in numbers. Is there a role for decidua in this context? The decidua as an extrathymic maturation site can be complementary to the thymic changes in at least two ways: (1) The need for positive selection ablated by cortex involution might be compensated for by extrathymic differentiation of T cells which will be primed on pregnancy-derived antigens in the decidual microenvironment and will allow to eliminate/silence fetus-reactive T cell clones. (2) Naive T cells generated in the medulla (e.g., regulatory cells) might be re-edited in the decidua thus adjusting their T-cell receptor repertoire to the paternal antigens. The γδT cells differentiated locally in decidua[33,46,47] will thus be specifically primed on the ongoing pregnancy.

Decidual αβ T Cells

Although a meaningful number (up to 19% of $CD45^+$ cells[35]) of αβT cells is found in human decidua, these cells have not been systemically studied so far. Representative electron micrographs of human decidual αβT cells stained with monoclonal antibodies for the TCRαβ are shown in Figure 8. As can be seen these cells highly express TCRαβ on the cell surface. Positive staining of the perinuclear envelope and the Golgi complex also demonstrates signs of

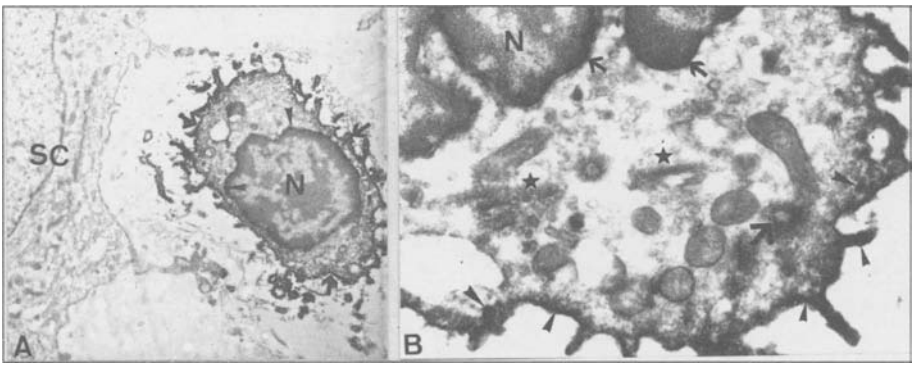

Figure 8. Immunoelectron microscopic features of αβT lymphocytes in the first trimester decidua stained with TCRαβ-specific mAb by an indirect immunoperoxidase method. No additional counterstaining was performed. A) Low power micrograph of αβT lymphocyte located close to stromal cell (SC). The cell surface of the lymphocyte forms numerous short processes and is definitely stained by the electron-dense reaction product showing the presence TCRαβ (arrows). Arrowheads point the positive staining of the perinuclear envelope. N= nucleus. Magnification, X 7,000. B) Area of the cytoplasm of an αβT cell. The cytoplasm contains two groups of the Golgi complex stained by the reaction product (stars). Thin arrows point at the staining of the perinuclear envelope. Thick arrow shows a positively stained multivesicular body. Note an intense surface staining (thin arrowheads) and the presence of cytoplasmic vesicles stained by the peroxidase reaction product (thick arrowheads). N= nucleus. Magnification, X 24,000.

active synthesis of TCR. Additionally, the cytoplasm contains multivesicular bodies (lysosomes) and vesicles stained by the peroxidase reaction product. There are obvious differences in TCR expression between αβ- and γδT cells at the ultrastructural level (compare Fig. 8 and Fig. 4). It is difficult now to interpret these differences since little is known about the fate of the TCR in resting and activated T cells.[54] Previous reports based on TCRαβ studies suggest that in resting T cells TCR constitutively cycles between the plasma membrane and the intracellular compartments with a low rate of degradation and de novo synthesis. TCRαβ engagement induces downregulation of the cell surface TCR levels and the degradation of the TCR in the lysosomal compartment that requires new synthesis of TCR complexes.[54] In contrast nothing is known so far about the recycling of TCRγδ.

Besides the CD3 complex, the decidual TCRαβ cells express either the CD4 molecule designating a T helper cell phenotype or the CD8 molecule, designating a suppressive/cytotoxic phenotype. In most studies of normal pregnancy, the number of the $CD8^+$ cells has been found to be slightly higher compared to the $CD4^+$ cells giving a CD4/CD8 ratio of approximately 1 or slightly decreased.[17,55] In recurrent abortions a decrease of $CD8^+$ cells and a strong increase of the CD4/CD8 ratio has been observed.[56] Both $CD4^+$ and $CD8^+$ cells express CD69, CD25, and HLA-DR showing that they are activated.[28] Our own investigation showed that a part of the decidual αβ T cells co-expressed the CD56 molecule and the great majority displayed several memory, activation and adhesion molecule markers. Interestingly within this population we found cells expressing the NK cell marker CD161 and CD 25, a marker of both effector and regulatory cells (LMN, unpublished data). Recently a novel murine cell population[57] of $CD4^-$ and $CD8^-$ uterine αβ T cells that simultaneously expressed B220, a marker predominantly found on B cells, has been described. A part of these cells expressed CD25 but did not produce TGF β and IL-10. Suppression of splenocyte proliferation was found in their presence. Moreover, these cells were found in the genital tract of both naive and pregnant mice ruling out pregnancy as an influencing factor for their presence and it was speculated that they might mature outside the thymus. Their nature and role in mice needs further investigation and their existence in humans has to be proven.

Table 3. General characteristics of NKT cells

T cell receptor type	TCRαβ (human Vα24JαQ; murine Vα14Jα281)
Natural killer receptor type	NKR-P1A (CD161) human, NK1.1 mice
Antigen presentation	CD1d
Natural ligand/s	unknown
Artificial ligands	lipids, lipoglycans
Phenotype	$CD4^-/CD8^-$ (most), $CD8^+$ (some)
Immunity affiliation	Innate immunity
Tissue distribution	Thymus, bone marrow, spleen, lymph nodes, peripheral blood, liver, intestinal mucosa, lung, endometrium/decidua, skin
Cytokine production	High and rapid: e.g., IL-4, INF-γ, TNF-α
Function	protect transplants fight infections kill tumors prevent autoimmune diseases

When discussing the decidual αβT cells, a very important distinction of two other lymphocyte subpopulations harbored within the αβ T cells should be made – the natural killer T (NKT) cells and the newly rediscovered $CD4^+/CD25^+$ αβT cells belonging to the innate or natural regulatory cells.

NKT Cells in Decidua

NKT cells are a population of TCRαβ lymphocytes that simultaneously express NK- and T cell-recognition receptors but do not possess the properties of MHC restriction, clonal expansion and immunologic memory of classical $CD4^+$ and $CD8^+$ cells. In the definition of the classical NKT cells three important features should be included: (1) A heavily biased usage of T-cell receptor chains (Vα24JαQ paired with Vβ11 in humans and Vα14Jα281 paired with Vβ8.2, Vβ7 or Vβ2 in mice) combined with the expression of the NK receptor NKR-P1A (CD161) in humans or NK1.1 in mice, (2) CD1d restriction and (3) high levels of cytokine production, particularly interleukin-4 (IL-4) and interferon γ (IFN–γ). In mice, these cells are commonly described as $NK1.1^+$αβTCR^+ cells. They are widely distributed in the body and play important role in infection protection, transplantation, tumor and autoimmunity prevention. The nature and properties of the NKT cells are summarized in Table 3.

The NKT cells are present in human and murine decidua. It has been shown that murine peri-implantation uterus contains an expanded population of $NK1.1^+$ $V\alpha14^+$ cells.[58,59] The murine decidual NKT population seems to be entirely $CD4^-/CD8^-$ and might recognize a fetal class I-like molecule other than CD1d.[60] Ito et al[59] reported that murine decidual $V\alpha14^+$ cells produced IFN-γ and TNF-α. In vivo stimulation with α-galactosylceramide, a ligand for $V\alpha14^+$ cells, provoked abortion suggesting involvement of decidual Vα NKT cells via TNF-α production and perforin-dependent killing that caused degeneration of the embryonic trophoblasts. This report is of a particular interest since it provides the first evidence that the trophoblast indeed is susceptible to direct attack by maternal lymphocytes in vivo implying that trophoblast survival during pregnancy might depend upon mechanisms that suppress effector immune response.

A homologue human population of $V\alpha24^+$ cells producing IL-4 and IFN-γ was described by Tsuda et al[61] in decidua and peripheral blood of pregnant women. One of the most thorough investigation of NKT cells at the feto-maternal interface performed by Boyson et al[62] showed that 0.48% of the human decidual $CD3^+$ cells were NKT cells, a frequency 10 times higher than that seen in peripheral blood. The decidual NKT cells produced high amounts of IFN-γ and thus exhibited a striking Th1 bias in contrast to the peripheral blood NKT cells that were Th2 biased and produced a high amount of IL-4. In our own investigation about 2% of

decidual αβT cells expressed the Vα24 chain and mRNA for the NK cell receptor CD161 (LMN, manuscript in preparation). Presence of CD1d on villous and extravillous trophoblast,[62] and CD1d mRNA transcripts in human trophoblastic and choriocarcinoma cell lines have been reported,[63] showing that this molecule, presenting a still unknown endogenous antigen to the NKT cells, is expressed in placenta. The role of the decidual NKT cells is currently not known but their general functions described above (see Table 3) make these cells very attractive players with potential to fulfil the specific requirements at the feto-maternal interface.

Regulatory T Cells in Decidua

Recently, there has been an explosion of interest among immunologists in a heterogeneous population of regulatory T cells (Treg) most of which co-express CD4 and CD25, and constitute a small number (5-10%) of peripheral $CD4^+$ αβT lymphocytes in normal naive mice and healthy humans.[64] $CD4^+$ Treg seems to suppress all forms of immune responses investigated to date both in humans and mice (reviewed in refs. 65-67 and Chapter 16).

Among $CD4^+$ Treg two different subsets can be distinguished by their specificity, development, mechanism of action and costimulatory signaling – natural and adaptive.[68] The characteristics of the natural Treg subsets are strikingly similar between mouse and man. The natural Treg that mature in the thymus, are present in all normal individuals and carry out their regulatory function during normal surveillance of self- and non-self antigens. The Treg are $CD4^+$ $TCR\alpha\beta^+$ cells that express high levels of CD25 in humans, and several activation markers like Cytotoxic T-lymphocyte Antigen 4 (CTLA-4) and Glucocorticoid-induced Tumor Necrosis Factor-related Receptor (GITR). Their peripheral homeostasis is controlled by signaling through CD28.[67] The forkhead/winged-helix transcription factor FoxP3 (scurfin in mice) is required for their development and function.[69-71] Although not completely clear, it has been proposed that natural Treg function by a cytokine-independent mechanism that presumably involves direct contact with responding T cells and antigen presenting cells. Moreover, the encounter of natural Treg with other T cells induces the latter to proliferate, to become suppressive and to acquire the ability to produce the inhibitory cytokines TGF-β and IL-10, a mechanism referred to as "infectious tolerance".[72,73] It has been argued that the natural Treg are most effective as suppressors of autoimmune responses in absence of inflammatory settings.[68]

The adaptive Treg are generated in the periphery from naive or resting T cells either from conventional $CD4^+$ $CD25^-$ cells or subsets of $CD4^+CD25^+$ cells under certain conditions requiring IL-2, TGF-β and IL-10, and antigen presentation by immature dendritic cells (DC).[73,74] The intensity of CD25 expression is variable and they might not require costimulation through CD28 for their development and functions. While the natural Treg are fully functional when they leave thymus, the adaptive Treg in the periphery might be triggered by the same antigen as effector T cells.[75] Adaptive Treg mediate their inhibitory activity by production of the immunosuppressive cytokines TGF-β (Th 3-type of response) and IL-10 (Tr-1 type of response). Besides $CD4^+$ T cells, a variety of adaptive Treg have been found among $CD8^+$-, $TCR\gamma\delta^+$-, and NKT cells.[76,77] The main induction sites of the adaptive Treg are settings with self-damaging inflammatory reactions to microbes or transplanted tissues or inflammatory autoimmune reactions that are more similar to infectious settings.

Various suggestions about the mode of action of adaptive Treg have been proposed including a cross-talk with the immature DC and induction of indoleamine 2,3-dioxygenase (IDO) in the DC. IDO is an IFN-γ inducible enzyme, that degrades tryptophan and plays a role in the control of immune responses and in peripheral tolerance induction. It has been shown that Treg interaction via CTLA-4 with the ligand B7 on the DC triggers the latter to produce IDO and inhibit T-cell proliferation.[78] Strikingly, inhibition of IDO has been shown to lead to abortion in mice.[79] A brief summary of the main characteristics of Treg is given in Table 4.

Immunologic tolerance towards the fetal allograft requires beyond doubt regulation of the maternal immune responses and hence the presence of regulatory cells. There are few publications about Treg in decidua. However, convincing reports show that both adaptive and natural

Table 4. Summary of the properties and subtypes of regulatory T lymphocytes

		Regulatory T Cell (Treg)
1.	**Definition**	A unique subpopulation of T cells with ability to suppress the functions of other immune cells and to play a critical role in the control of the immune response and the generation and maintenance of tolerance.
2.	**Properties**	heterogeneous group of lymphocytes exist in low numbers respond poorly to stimulation through TCR unique and diverse mechanisms of action produce the regulatory cytokines TGF-β and/or IL-10
3.	**Some subtypes**	
	by site of development	innate/natural- develop in the thymus adaptive/inducible- develop in the periphery
	by phenotype	*$CD4^+CD25^+$* *$FOX\ P3^+$* *$CTLA4^+$* *$GITR^+$* *$CD4^+CD45RB^+$* *$CD8^+$* *$TCR\gamma\delta^+$* *NKT cells*
	by cytokine profile	*Th3 cells*: differentiate from naive $CD4^+$ or $CD8^+$ cells under the influence of TGF-β produce TGF-β > IL-10, varying IL-4 *Tr1 cells*: differentiatie from naive $CD4^+$ or $CD8^+$ cells under the influence of IL-10 produce IL-10 > TGF-β, no IL-4

Treg are present in pregnant uterus and play an important role in the creation of local and systemic maternal immunotolerance towards the fetus.[80-83]

Aluvihare et al[81] have investigated natural Treg in murine pregnancy. Treg cells were required for the maternal immune system to tolerate the fetal allograft and there was an active recruitment and/or expansion of $CD4^+CD25^+$ Treg locally in the murine pregnant uterus and systemically in the spleen and lymph nodes of the pregnant mice. The generalized expansion of the Treg suggested that suppression of anti-fetal responses might also occur within the draining lymph nodes thus preventing activation of potentially dangerous effector cells. Importantly, the expansion of the natural Treg was not driven by fetal alloantigens. Instead it might be due to a hormonal modulation of the maternal immune system.[51,52,84,85] Human decidua from first trimester[82] and term pregnancy[83] was shown to contain $CD4^+CD25^+$ Treg. In early pregnancy, these cells comprised about 20% of all decidual $CD4^+$ cells, co-expressed the CTLA-4 molecule, and were able to suppress proliferation of autologous anti-CD3 mAb stimulated T cells. The suppression required cell-cell contact. Decidual specimens from recurrent abortions had significantly reduced amounts of $CD4^+CD25^{+bright}$ Treg compared to specimens from induced abortions suggesting that these cells might contribute to the maternal immune tolerance of the conceptus.[82] According to our data human normal early pregnancy decidua contains about 15-20 % $CD25^{+bright}$ cells within the $CD4^+$ αβT cells. Moreover, these cells expressed mRNA for the Treg marker FoxP3 (LMN, unpublished). In term decidua about 14% of the $CD4^+$ population was found positive for CD25. Around half of these cells expressed intracellular CTLA-4 as well as GITR and OX-40.[83]

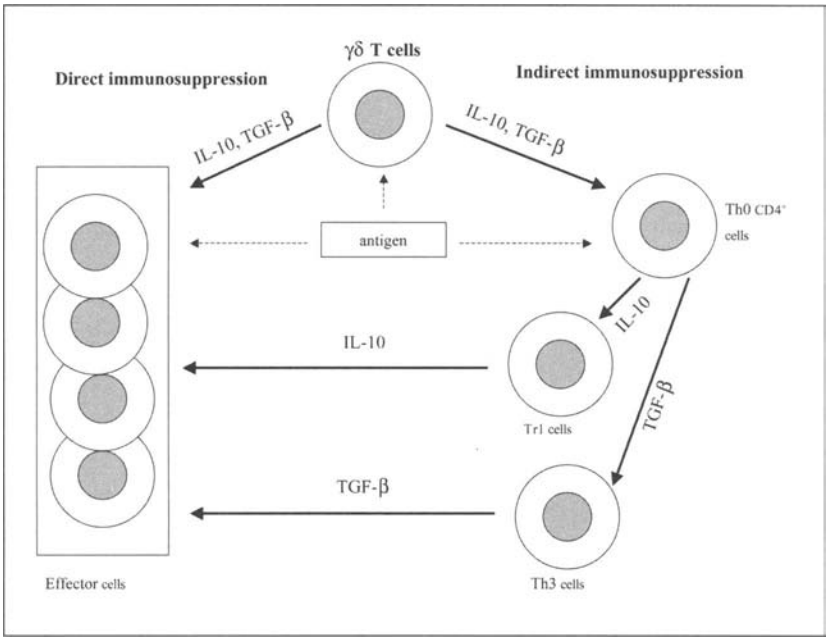

Figure 9. Schematic presentation of two possible mechanisms by which decidual γδT cells could induce local tolerance. Under influence of pregnancy associated antigen(s) γδT cells start producing IL-10 and TGF-β. These cytokines could directly inhibit the effector cells (direct immunosuppression). On the other hand, these cytokines could create an environment for generation of Tr1 and/or Th3 cells, which in their turn secrete IL-10 and TGF-β and suppress the effector cells (indirect immunosuppression). Reprinted with permission from ref. 10.

Our studies of decidual γδT cells from human early normal pregnancy[33,80] show that these cells mature locally in decidua, express CTLA-4 (Fig. 3) and have a cytokine mRNA profile of high IL-10 production followed by significant but lower TGF-β mRNA expression. In addition our preliminary results indicate that these cells express mRNA for FoxP3 (LMN, unpublished). Thus, the phenotype of the decidual γδT cells and their cytokine profile is consistent with adaptive Tr1 type of regulatory cells and suggests a regulatory function at the feto-maternal interface exerted through direct immunosuppression and/or generation of other immunosuppressive cells as illustrated in Figure 9.[80]

Although a lot more is left to be done to elucidate all subtypes of decidual Treg and their properties and function, all the facts so far support the idea that immunoregulation is the key feature and the most essential function of the decidual T cells. The suppression of maternal effector cells, potentially aggressive to the fetus, is a core question and Treg play a crucial role in its solution. Pregnancy is a physiological state that evokes a hormone-related central response taking place in thymus that generates natural Treg and an alloantigen-related peripheral response taking place in decidua that generates adaptive Treg. These two responses are interconnected and work in concert to contribute to the maternal immunotolerance towards the fetus which is the essential piece in the jigsaw puzzle molding a successful pregnancy. Combining the known data published by others and us about immunotolerance at the feto-maternal interface we present a hypothetical model (Fig. 10) illustrating the origin of decidual Treg and their contribution to the suppression of alloresponses during pregnancy. Two main pathways of regulation, started by two different responses are delineated-hormone-related response generating natural Treg and alloantigen (pregnancy)-related response generating adaptive Treg. In the first pathway under the hormonal influence of pregnancy, thymic expansion of natural

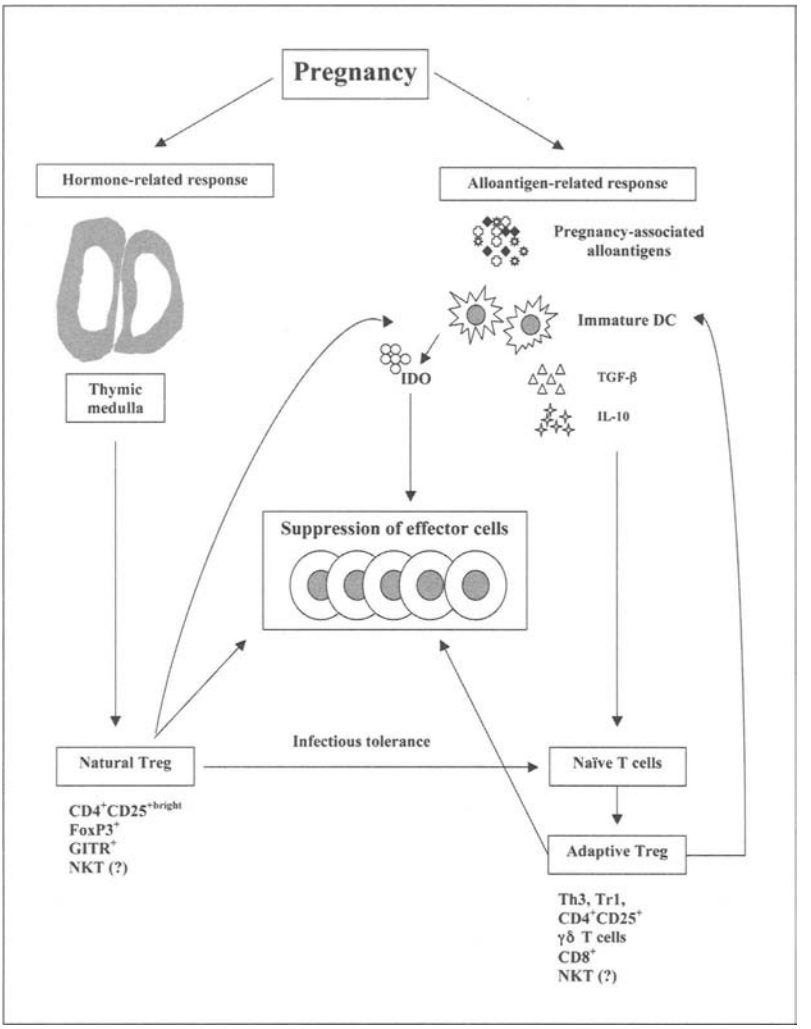

Figure 10. A hypothetical model for the regulatory T lymphocyte function in pregnancy. DC= dendritic cells, IDO= indolamine 2,3-dioxygenase, Treg= regulatory T cells.

$CD4^+CD25^+$ Treg and probably regulatory NKT cells takes place. These cells acquire then a suppressive ability and can act in two ways (1) on the effector cells as Treg themselves or (2) through influence on naive T cells, which become adaptive Treg. In the second pathway fetus-related alloantigens are presented by immature DC in an immunosuppressive cytokine milieu of IL-10 and TGF-β. Naive T cells stimulated by immature DC under these circumstances are primed to become adaptive Treg. Both natural and adaptive Treg can stimulate DC to produce IDO and thus inhibit proliferation and induce apoptosis of the effector cells. A considerable amount of reported evidence supports this hypothesis – hormonally induced thymic changes,[51,52] the presence of immature DC decidua,[86] the existence of IL-10/TGF-β cytokine milieu,[80] a well-documented IDO production by DC and macrophages at the feto-maternal interface[87,88] and last but not least recent and convincing reports[80-83] of the presence of Treg of both types in human and murine pregnancy.

Conclusions

The core question how a mammalian fetus escapes the immunologic attack of the maternal immune system has puzzled immunologists for years. The mechanisms of immunotolerance during pregnancy are the main focus of research in immunology of reproduction. Despite this research interest, one field has been seriously neglected – studies of the decidual T cells. There are several reasons for this unfortunate bias – one major reason being the fact that the decidual T cells are fewer compared to the dominating the $CD56^{+bright}$ NK-like cells. Other reasons for the negligence have been the difficulties to isolate and characterize the T cells phenotypically, due to their low number and weak expression of surface markers,[33,89] and functionally, due to their inertness in in vitro functional assays. Published discrepancies,[24,25,33,89] due to the differences in the antibodies used have also contributed to the low interest in these cells. The hegemony of the $CD56^{+bright}$ cell research has been so utter and complete in immunology of reproduction that still recent statements have been made that the T cells in decidua are simple by-standers with no purpose or function in pregnancy at all.[90,91] No statement can be more wrong and no opinion more biased. The comparatively few serious studies about the phenotype, distribution and function of decidual T lymphocytes are summarized in this chapter. There is no doubt that the T cells are permanent constituents of decidua in all mammals so far investigated. What do we know about the decidual T cells at present? The T cell population in decidua comprises between 15-20% of the total number of leukocytes,[35] persists in numbers and is even enriched compared to the $CD56^{+bright}$ cells throughout the pregnancy. The decidual T cells have a proliferative ability as tested by mitogenic lectins and anti CD-3 antibodies but their allospecific responses are inhibited. The decidual T lymphocytes harbor a variety of T cells – γδT cells,[10] conventional αβT cells, NKT cells and last but not least the recently revived Treg cells. The decidual γδT cells mature and divide locally, possess a cytotoxic potency and prime on the ongoing pregnancy.[10] Through their cytokine production they act as a Tr1 Treg and actively contribute to the immunosuppressive cytokine milieu in decidua that promotes the generation of adaptive Treg and down regulates potentially dangerous anti-fetal responses. NKT cells are present at the feto-maternal interface and are the first lymphoid cells documented to be able to recognize and react on fetal trophoblast, a fact logically assumed but difficult to prove in an experimental system. The first reports of natural and adaptive decidual Treg present have just started to open a new path towards the understanding of the immunotolerance of the fetus. What is the future of decidual T cell research? The answer is: "Imperative, worthwhile, useful, necessary, bright and extremely exciting!" The new era of revival of Treg opens new opportunities in decidual T cell research. Treg research in pregnancy is just at the beginning. Thanks to that decidual T cells finally get the attention they deserve. More and different decidual regulatory cell subpopulations will be characterized,[92] and their role and function studied. Of all functions of the decidual T cells described above, the one of key importance is the immunoregulatory. It might be the so-longtime missing masterpiece in the jigsaw puzzle of mechanisms that mould the frame of a successful pregnancy.

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CHAPTER 19

Trophoblast Cells as Immune Regulators

Gil Mor and Vikki M. Abrahams

Abstract

Medawar, in the early 1950s, recognized for the first time, the unique immunology of the maternal-fetal interface and its potential relevance for transplantation. In his original work, he described the "fetal allograft analogy" whereby the fetus may be viewed as a semi-allogeneic conceptus that has evaded rejection by the maternal immune system. Although numerous hypotheses have been proposed to prove this observation, none have demonstrated that the maternal immune system is antagonist to the invading trophoblast. In the present manuscript we have reviewed recent studies demonstrating the expression and function of TLRs in trophoblast cells and based on this data we propose an alternative view for maternal-fetal immune interactions.

Introduction

Over fifty years ago the renowned transplant immunologist, Sir Peter Medawar, proposed the paradigm of why the fetus, as a semi-allograft, is not rejected by the maternal immune system.[1] Subsequent studies demonstrated the presence of an active maternal immune system at the implantation site and this provided evidence to support Medawar's original notion. As a result, investigators began to pursue the mechanisms by which the fetus might escape such maternal immune surveillance. Furthermore, alterations in these pathways in pregnancy complications, such as recurrent abortion and preeclampsia, where the immune system is thought to play a central role, have been used as further evidence for the Medawar hypothesis. As a consequence, since Dr. Medawar's original observation, numerous studies have been performed in order to explain this paradigm, many of which have been centered on how the fetus and placenta fight against an active and aggressive maternal immune system. While varied hypotheses have been proposed in order to explain how the maternal immune system might be neutralized during normal pregnancy (see ref. 2 for review of the different hypotheses); none, however, have been able to convincingly demonstrate that in normal pregnancy there exists an immunological attack on the fetus.

The Immunology of Pregnancy

The finding of macrophages and neutrophils at the implantation site as early as the first week of implantation,[3] as well as the high numbers of immune cells present at the maternal-fetal interface throughout pregnancy, have been taken as conclusive proof that the maternal immune system responds to the allograft fetus. Normal pregnancy is characterized by immune cells present at the maternal-fetal interface and it is the innate immune system that dominates the early pregnant decidua. 70% of decidual leukocytes are natural killer (NK) cells, 20-25%

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are macrophages and approximately 1.7% are dendritic cells.[4-6] From the adaptive immune system, B cells are absent, but T lymphocytes constitute about 1-3% of the decidual immune cells during the first trimester of pregnancy.[7] Decidual NK cells are phenotypically distinct ($CD56^{bright}$, $CD16^{-}$), and unlike their circulating equivalents, uterine NK cells have a morphology similar to large granular lymphocytes and display low cytotoxicity.[8] It is thought that these cells do, however, migrate in from the periphery.[9,10] During the first trimester, NK cells infiltrate the decidua and accumulate around the invading trophoblast cells, however, as gestation proceeds these innate immune cells progressively vanish and are absent at term.[11] In contrast, decidual macrophages maintain their presence throughout gestation.[4] Decidual macrophages are found located in the decidua basalis and paritalis and are densely distributed beneath the uterine epithelium that surrounds the fetus.[3] These innate immune cells are also in close contact with extravillous trophoblasts. The observed migration of immune cells into the maternal-fetal interface during normal pregnancy, coupled with their close proximity to the invading trophoblast cells, prompted Medawar's hypothesis to be altered; yet strengthened. Thus, the maternal immune system is not responding to the fetus, but instead to the trophoblast.

Pregnancy Represents an Allograft

Medawar, in the early 1950s, recognized for the first time the unique immunology of the maternal-fetal interface and its potential relevance for transplantation. In his original work, he described the "fetal allograft analogy" whereby the fetus may be viewed as a semiallogeneic conceptus that has evaded rejection by the maternal immune system.[1] Subsequently, a number of mechanisms were proposed to account for this lack of fetal rejection. The different hypotheses can be summarized into five main concepts: (i) The mechanical barrier effect of the trophoblast; (ii) Systemic suppression of the maternal immune system during pregnancy; (iii) The absence of classical MHC class I molecules in the trophoblast; (iv) A cytokine shift; and more recently (v) Localized immune suppression. Unfortunately, none of them can adequately explain or prove the existence of the fetal allograft analogy.[2] Indeed, we know today that the trophoblast does not provide a mechanical barrier, since there is evidence for the trafficking of cells in both directions across the maternal-fetal interface. This includes the migration of maternal cells into the fetus,[12] and the presence of fetal cells in the maternal circulation.[13-15] Indeed, this is the case in almost all the immune privileged tissues, including the brain's blood brain barrier. Conclusive evidence has shown that immune cells circulate through all the parts of the brain[16] indicating that mechanical barriers do not deter the migration of leukocytes into supposedly immune privileged sites.[17] The hypothesis of systemic immune suppression has been challenged by recent studies clearly demonstrating that maternal anti-viral immunity is not affected by pregnancy. The clearest observation is that HIV^{+} pregnant women do not suffer from AIDS-like disease argues against the existence of such nonspecific immune suppression during pregnancy.[18] The expression of HLA-G, a monomorphic, trophoblast-specific HLA class I molecule was used to explain why trophoblast survives, despite the presence of abundant decidual NK cells. According to this hypothesis, the HLA-G molecule acted as a surrogate "self" class I molecule thus preventing NK cell killing.[19,20] However, more recent studies by several groups have not supported this hypothesis.[21,22] The definition of pregnancy as a "TH2 environment" was originally enthusiastically embraced and numerous studies tried to prove and support this hypothesis. It is now becoming increasingly clear that the TH1/TH2 nomenclature is a hindrance beyond $CD4^{+}$ T cell functions; and while there are strong evidences that anti-inflammatory cytokines, such as IL-10, are relevant for the success of pregnancy, it is also true that pro-inflammatory cytokines, such as IL-6 and IL-8, are produced during and necessary for normal pregnancy. Lastly, the idea that the trophoblast creates an immune privileged microenvironment by eliminating immune cells that pose a potential threat, specifically through the Fas/Fas ligand system,[23-25] has been challenged by the lack of an immune response or fetal rejection in mice lacking Fas (*lpr*) or Fas ligand (*gld*).[26] Our recent studies indicate that Fas ligand (FasL) is not expressed at the cell surface membrane of trophoblast cells, but is instead

secreted via microvesicles to act on Fas expressing cells at locations away from the implantation site.[27] The role of this functional secreted FasL is not fully understood and is under investigation. More recently, the role of IDO[28] and T regulatory cells (Trg)[29] have been proposed as potential mechanisms for the immunological escape of the fetus. Numerous groups are pursuing this hypothesis, and their results will determine whether this theory is valid.

In reviewing these different hypotheses we have observed that the field of Reproductive Immunology has always followed mainstream immunology; translating the findings from transplantation to explain the immunology of the maternal-fetal relationship. So far, all of these ideas have failed to conclusively prove the principle of semi-allograft acceptance by the mother and has produced confusion on the role of the immune system during pregnancy. Therefore, it is necessary that we reevaluate the basic question of reproductive immunology: Does the fetal/placental unit truly act as an allograft that is in continual conflict with the maternal immune system?

Challenging the Medawar Hypothesis

Transplantation vs. Implantation

Medawar's observation was based on the assumption that the placenta is a "piece of skin" with paternal proteins, which under normal immunological conditions, should be rejected. However, the placenta is more than just a transplanted organ. Our knowledge of placental biology has significantly increased over the last 50 years. We now know that the placenta is a complex organ, which has evolved from the original "egg cover". Pregnancy and implantation, contrary to "graft implants", has been taking place for more than 100,000 years. Therefore, from an evolutionary point of view it is difficult to conceive that the placenta and the maternal immune system still maintain an antagonistic status. Furthermore, as our understanding of role of the immune cells at the implantation site increases, we learn that many of these leukocytes are present for the protection and maintenance of the pregnancy, rather its rejection. Therefore, we propose that the trophoblast and the maternal immune system have evolved and established a cooperative status, helping each other against common enemies, such as infectious microorganisms. In the present review we will discuss some of the evidences suggesting that the immune system is critical for pregnancy success and that the trophoblast itself may function as a normal component of the innate immune system, so that together they can defend the maternal-fetal interface against invading pathogens and, as we will discuss, possibly take advantage of commensal microbes.

Infection and Pregnancy

Clinical studies have shown a strong association between certain pregnancy complications and intrauterine infections,[30,31] suggesting that the innate immune response can affect the outcome of a pregnancy. Preeclampsia and intrauterine growth restriction (IUGR) are both thought to be associated with infection[32-34] and a link between preterm labor and intrauterine infections is now well established. Indeed, infections have been reported as responsible for up to 40% of preterm labor cases.[35] Furthermore, 80% of preterm deliveries occurring at less than 30 weeks of gestation have evidence of infection,[36] suggesting that an intrauterine infection may occur early in pregnancy, preceding such pregnancy complications.[30] Infection as a mediator of inflammation, therefore, represents an important and frequent mechanism of disease. However, inflammation is also necessary for normal implantation and parturition. Implantation is characterized by the production of chemokines, pro-inflammatory cytokines and other inflammatory mediators.[37] Blockage of this inflammatory process in rodents results in implantation defects.[38] In contrast, animal models of pregnancy complications demonstrate that inflammation is often an underlying cause (reviewed in Ref. 39). Understanding how the trophoblast and the maternal immune system regulate inflammation represents the core for understanding maternal-fetal immune interactions.

The Trophoblast and Implantation

The trophoblast is not a classical differentiated epithelial cell. Instead, it is an embryonic stem cell with the outstanding capacity of adaptation to changing environments, tissue remodeling and organ development. Embryonic implantation consists of three consecutive phases; apposition, adhesion and invasion, and in each of these steps the trophoblast will confront different cell types and microenvironments. The success of the pregnancy depends on how well the trophoblast responds and adapts to each of these stages. Starting from the process of cell attachment to the lumen of the uterus, followed by invasion into the decidua and finally to transformation of the spiral arteries, the invading trophoblast requires a high capacity to communicate with its cellular environment.

As already discussed, the human decidua contains a large number of immune competent cells such as macrophages, NK cells and T cells. These leukocytes, as well as the decidual stromal cells themselves, are capable of producing soluble cytokines and hormones, all of which are necessary for both the regulation of immune responses and the growth and development of the placenta. The appropriate communication between all these cellular components at the fetal-maternal interface is crucial for successful reproduction. In addition, the upper female reproductive tract including the uterine lumen, is often exposed to commensal bacteria and bacterial products from the lower tract.[40,41] Such microflora may interact with the external layer of the blastocyst while it is at the luminal surface. Moreover, as the blastocyst invades the endometrium, bacterium may gain access into this maternal compartment. Thus, for successful implantation, the invading trophoblast must recognize its new environment consisting of maternal cellular /soluble components and foreign microbes, and consequently respond by sending out the appropriate signals that will facilitate its adaptation and growth. Several questions arise from these observations and will be discussed thereafter:

- How does the blastocyst respond to the presence of bacteria in the uterine lumen?
- How do the trophoblast and the maternal immune system prevent the invasion of bacteria from the uterine lumen into the decidual stroma during implantation?
- How does the trophoblast communicate with the maternal immune system to prevent pathogenic bacteria invading the uterus?

How the Trophoblast Recognizes and Responds to Microbes

The signaling loop that mediates innate immune responses to microorganisms is based on the sensing of conserved structural motifs, known as pathogen-associated molecular patterns (PAMPs) that are specifically expressed only by microorganisms.[42] PAMPs include bacterial components such as lipopolysaccharide (LPS) and peptidoglycan (PGN), and viral components such as dsRNA. These motifs, expressed by both commensal and pathogenic microorganisms, are recognized by pattern recognition receptors (PRRs). The best known PRRs are the toll-like receptors (TLRs), which are expressed by cells of the myeloid and lymphoid lineages, as well as by epithelial and endothelial cells.[43,44]

Toll-Like Receptors (TLR)

All living organisms are constantly exposed to microorganisms present in the environment and invasion by such foreign bodies must be controlled. Therefore, the proper recognition and response towards potentially pathogenic microorganisms must be in place. Recent studies have shown that the innate immune system has a greater degree of specificity that was previously thought and that it is highly developed in its ability to discriminate between self and infectious nonself. This discrimination relies, to a great extent, on a family of evolutionary conserved receptors, known as TLRs, which have a critical role in early host defenses against invading pathogens.

Toll-like receptors are transmembrane proteins, which have an extracellular domain containing leucine-rich repeat motifs. Each receptor differs in their ligand specificity. So while individually, TLRs respond to limited ligands, collectively the family of TLRs can respond to a

wide range of proteins associated with bacteria, viruses, fungi and parasites. TLR-4 was the first human Toll-like receptor to be identified[45] and was subsequently found to be the specific receptor for the recognition of LPS.[46-48] To date ten members of the TLR family has been identified in humans. TLRs 1, 2, 4, 5 and 6 and 9 appear to specialize in the recognition of mainly bacterial products, while TLRs 3, 7 and 8, in contrast, specialize in viral detection. While extracellularly, each TLR is distinct in their specificity, all receptors signal through a common pathway. Toll-like receptors have an intracellular domain which is highly homologous to the type-1 Interleukin-1 receptor (IL-1R) and is known as the Toll/IL-1R homology region (TIR).[49] Both TLR and the IL-1R recruit and interact with the adapter signaling protein, myeloid differentiation factor 88 (MyD88).[50] Following ligation of a TLR by its ligand, MyD88 becomes associated with the intracellular domain of the receptor through a TIR-TIR interaction.[51-54] In turn, MyD88 through its DD recruits and activates the DD-containing serine/threonine kinase, IL-1R associated kinase (IRAK).[52] Subsequent downstream activation of the NF-κB and MAP kinase signaling pathways occurs through activation of a kinase cascade and results in an inflammatory response characterized by the production of cytokines and chemokines. In addition, NF-κB and JNK activation induced by TLR-3 and TLR-4, can also occur via MyD88-independent pathways[55] which can stimulate the production of type I interferons (IFNα and IFNβ) and trigger the expression of IFN-inducible genes.

Toll-Like Receptors, Commensal Microbes and Pregnancy

The upper reproductive tract was originally thought to be a sterile environment that was infrequently exposed to bacteria present in the ecto-cervix and vagina. However, recent studies indicate that epithelial cells of the female reproductive tract are exposed to bacteria through peristaltic contractions at a frequency not previously appreciated.[37] Following mating, viable bacteria can also be transported with the semen towards the uterine lumen.[56] In the nonpregnant uterus the endometrial epithelium functions as a protective barrier against such microorganisms present throughout the reproductive tract. However, it would be incorrect to suggest that the reproductive tract is in a constant state of low inflammation. On the contrary, the uterus and fallopian tubes have a relatively low incidence of chronic infections, suggesting the existence of physiological mechanisms that control inflammatory responses towards commensal bacteria. Several studies have identified TLR expression throughout the female reproductive tract (FRT).[57] Pioli et al (2004) have shown expression of TLR1 - TLR-6, MyD88, MD-2, and CD14 in both lower and upper FRT tissue.[58] A recent study of in vivo expression of Toll-like receptors throughout the FRT epithelium reported expression of TLR-1, TLR-2, TLR-3, TLR-5 and TLR-6 in the lower tract, with TLR-4 found only on the epithelium of endocervical and uterine tissue and endometrial glands.[59] Additionally, this study reported a secreted form of TLR-4 from endocervical glands. All these data indicate that the female reproductive tract, upon activation of TLRs, may produce cytokines and chemokines that can regulate the differentiation, maturation and recruitment of leukocytes in the underlying stroma.

The window of implantation or opportunity for the embryo to adhere to the endometrium is defined by specific changes in the expression of epithelial integrins and mucins, allowing close apposition between the blastocyst and the luminal surface.[60,61] The expression of these adhesion molecules is cytokine dependent and thought to be induced by seminal plasma.[62,63] Bacterial products transported through the reproductive tract in association with seminal plasma may constitute such pro-inflammatory stimuli needed for apposition of the blastocyst. It is, therefore, tempting to speculate that this bacterial stimulus may be essential for implantation. There are numerous observations both in humans and animals to support the priming role of the semen,[64] although the focus of those studies have been on cytokines or factors present in the seminal fluid.[62,64] An alternative priming factor may be from the commensal bacteria present in the vagina that may be carried into the upper tract by the seminal fluid. Once in the uterus, such bacterium may be recognized by the uterine epithelium through TLRs and induce this epithelial layer to produce cytokines and chemokines. Therefore, the presence of commensal

bacteria within the uterine lumen may be essential for successful implantation in both it's priming capacity and also in it's ability to limit the growth of more virulent microbes.

The priming effect of commensal microbes may not be limited only to the uterine epithelia, but also to the developing blastocyst as it passes through the oviduct and into the uterus prior to, and during, implantation (Fig. 1). The trophoectoderm, the external layer of the embryo, may also recognize bacterial products through TLRs and respond to them through the production of cytokines.

Indeed, trophoblast cells throughout the whole of pregnancy express TLRs. Initial studies have reported mRNA expression of TLR-1 - TLR-10,[65] as well as protein expression of TLR-2 and TLR-4,[66,67] in term placenta. Since then we have observed that in first trimester placental tissues, functional TLR-2 and TLR-4 are highly expressed in the villous cytotrophoblast and extravillous trophoblast populations.[68] Interestingly, while third trimester trophoblast cells express TLR-2 and TLR-4, these receptors are not expressed by first trimester syncytiotrophoblast cells.[68] Together these findings have demonstrated that trophoblast cells expressing TLRs may respond to bacterial products.[66,68] How then can the trophoblast tolerate LPS from commensal gram-negative bacteria but also react to the same LPS from a pathogen? One potential explanation is the compartmentalization of TLR-4. This compartmentalization can be either intracellular localization or cell type specific. In the first case, we found that, contrary to the classical membrane expression of TLR-4 on many of innate immune cells, such as macrophages, trophoblast cells express TLR mainly in the cytoplasm; suggesting that these cells may recognize LPS associated with bacteria that have been internalized. We have also found that the

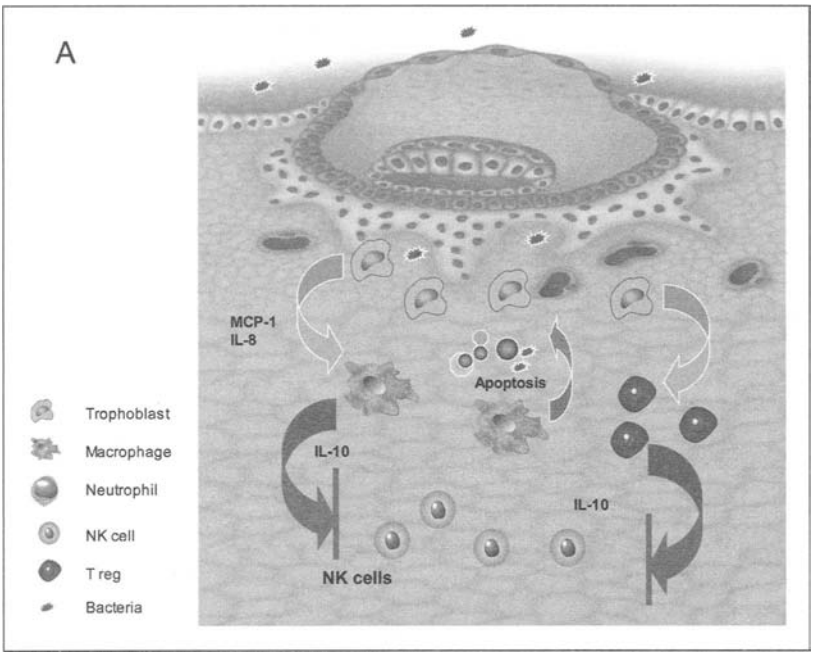

Figure 1. Recognition and response: The trophoblast recognizes, through TLRs, microbes and the cellular components at the implantation site and responds to them through the production of cytokines and chemokines. A) During normal implantation, trophoblast cells secrete chemokines, such as MCP-1 and IL-8, promoting the recruitment of macrophages and NK cells which then protect the trophoblast against infection and facilitate trophoblast invasion. Figure and legend continued on next page.

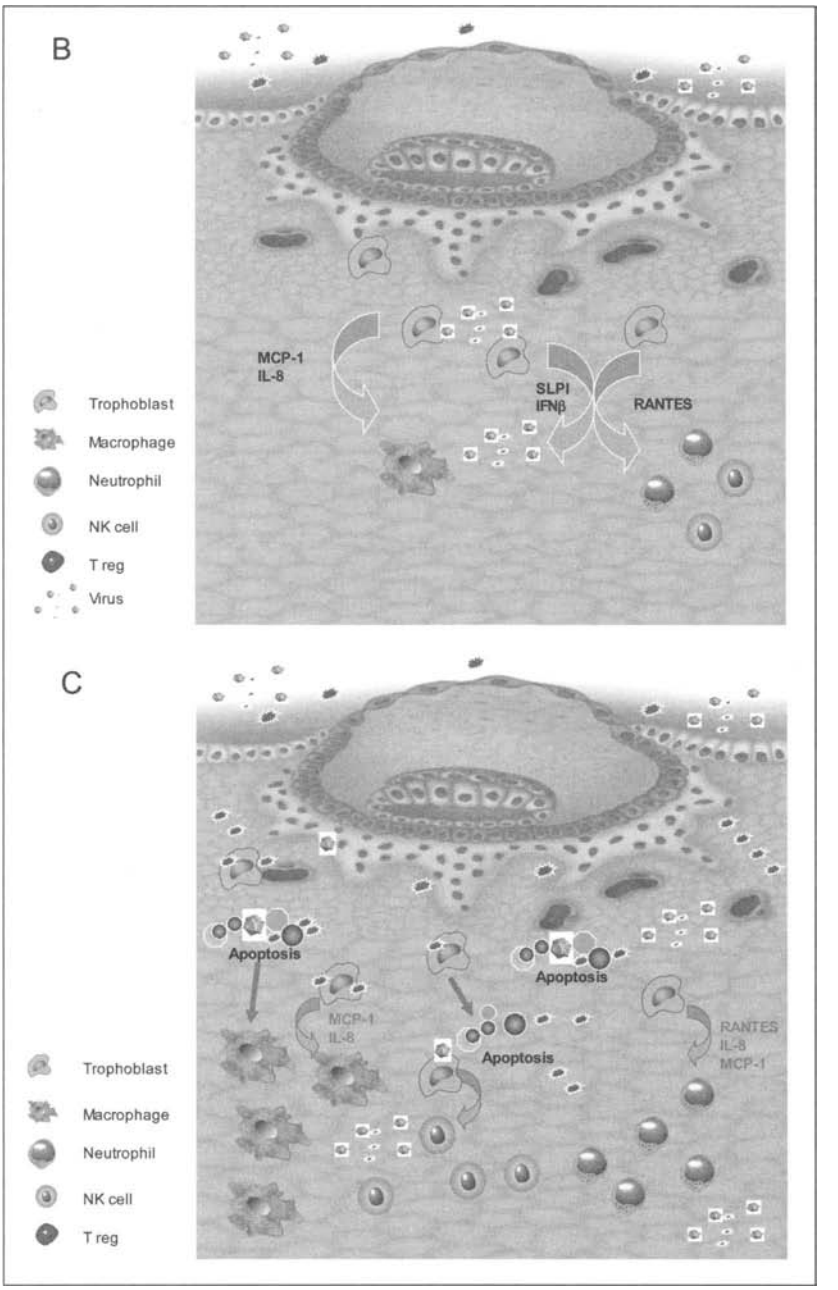

Figure 1. Continued. B) Anti-viral properties of the trophoblast. Trophoblast cells, through TLRs, recognize viral products and actively initiate an anti-viral response by producing interferons and anti-viral peptides. C) In disease, the cross talk between the trophoblast and the maternal immune system is broken. This may arise from unrestrained infection or an excessive inflammatory response due to macrophage, NK cell and neutrophil activation. Both may result in elevated trophoblast apoptosis.

expression of TLR is cell type specific, according to its tissue localization. In first trimester placentas, the syncythiotrophboast (the outer layer of the villi) is negative for TLR-2 and TLR-4, while the internal layer, the cytotrophoblast, is positive for both receptors.[68] The lack of TLR expression by the outer trophoblast layer during the first trimester is analogous to studies of mucosal epithelial cells of the intestinal tract, which have been shown to express TLR-5 only on their basolateral side.[69] These cells will only respond to a bacterium that has invaded the basolateral compartment from the apical side. Since a pathogen is characterized as a microorganism that breaches certain physical barriers, these observations have helped to explain how an immune response can be mounted against pathogenic, but not commensal bacteria. Similarly, a microorganism will only be a threat to the fetus if the TLR-negative syncytiotrophobast cell layer is breached and the pathogen has entered either the decidual or the placental villous compartments. Therefore, the placenta may distinguish between pathogenic and commensal microorganisms during pregnancy. Once an infection has gained access to the TLR positive trophoblast cells, a response may be mounted. As described below, the type of pathogen and, therefore, the specific receptor activated may have a significant impact on the type of response generated by the cells of the placenta.

Cross Talk between the Trophoblast and the Innate Immune System

TLRs and the Regulation of an Immune Response

A fundamental feature of the immune system is to protect the host from foreign bodies or abnormal cells. This function resides on the innate immune system's capacity to coordinate cell migration for surveillance, to recognize and respond to invading pathogens, and to facilitate the efficient clearance of dying cells. The recognition of pathogens or inflammatory signals at the site of an infection by the innate immune system triggers the process of cell migration. Innate recognition of PAMPs through TLRs initiates an inflammatory response, characterized by the recruitment of immune cells to the site of infection in order to augment microbial killing and halt spread. Cell migration from the peripheral blood into inflamed tissues involves a tightly controlled sequence of events, which are mediated by the two type of signals; chemokines and cell surface adhesion molecules. Activation of TLRs by microbial products induces the expression of chemokine and their receptors which in turn regulate immune cell migration to the sites of inflammation.[70] Key inflammatory chemokines produced during acute microbial infections include interleukin 8 (IL-8), growth related oncogene α (GRO-α), monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein 1α (MIP-1α), and RANTES (Regulated Upon Activation Normal T cell Expressed and Secreted). The trophoblast is able to produce all these chemokines; some of them constitutively, such as IL-8, MIP-1α, MCP-1 and GRO-α, and others only following activation of certain TLRs, such as RANTES.[71-73] Chemokine production is critical for implantation in order to facilitate immune cell recruitment into the decidua for host defense, but also for what we, and others, believe is essential immunological support during pregnancy.[74,75]

Trophoblast Cells Regulates Macrophage Migration

Using a two-chamber migration system, we, and others, have observed that trophoblast cells are able to chemoattract monocytes and NK cells (Fig. 2).[72,73] These observations have shed new light on the cross-talk between trophoblast cells and the immune system. Instead of the maternal immune system responding to the invading trophoblast as foreign, it appears that the trophoblast, under normal conditions, is playing a central role in regulating leukocyte migration into the decidua, suggesting that the immune cells themselves are important for pregnancy. In vivo models suggest that the presence of NK cells within the endometrium is necessary for successful implantation[76,77] and it has been postulated that NK cells play a role in the decidualization of the endometrium.[78] Similarly, macrophages at the implantation site and throughout gestation are thought to benefit pregnancy. Decidual macrophages may efficiently

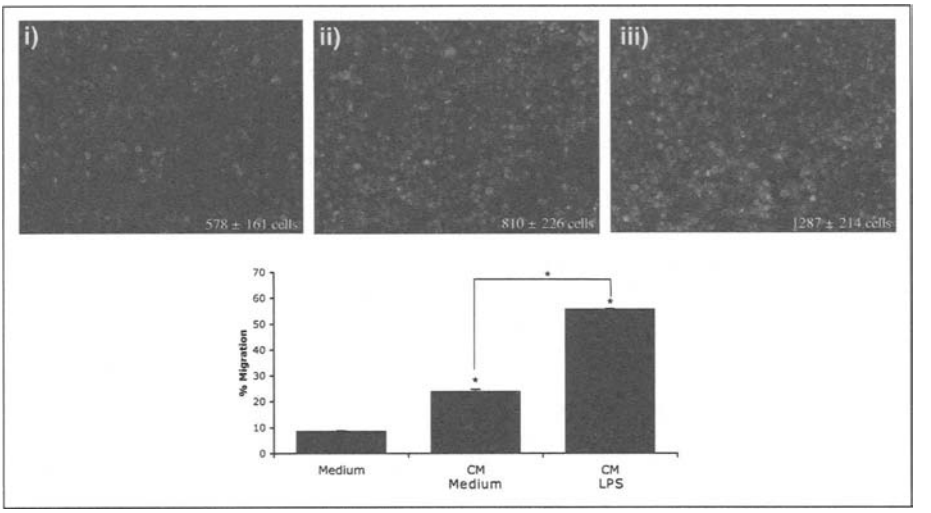

Figure 2. Monocytes migrate towards LPS-stimulated first trimester trophoblast cells. Using a two-chamber migration system, we observed significant monocyte transmigration towards trophoblast cells (panel ii), when compared with random monocyte migration (panel i). Moreover, the pretreatment of trophoblast cells with LPS (10/mu/g/ml) further enhanced monocyte chemotaxis (panel iii). Bar chart shows the quantification for each group: Monocyte transmigration towards the conditioned media from untreated (CM Medium) or LPS stimulated (CM LPS) trophoblast cells (*$p<0.001$).

clear apoptotic cells, thus maintaining tissue homeostasis. Macrophages may also provide the trophoblast with soluble factors that will stimulate their growth and differentiation, as well as promoting the invasion and transformation of the spiral arteries.[74,75,79]

As the trophoblast breaches the epithelial surface of the lumen during implantation, it may allow access of bacteria into the uterine stroma. While, acute inflammation is necessary for implantation, it is also critical to avoid chronic inflammatory responses that may be triggered by excessive bacterial invasion. To this purpose, we hypothesize that the trophoblast itself, promotes a local innate immune responses towards microbes within the stroma, thereby increasing protection (Fig. 1). Again using the in vitro migration system, upon ligation of TLR-4 by bacterial LPS, chemokine production by trophoblast cells is significantly increased and this further enhances monocyte migration[73] (Fig. 2). Furthermore, the chemokine response following activation is differentially regulated depending upon the stimuli. When trophoblast cells are exposed to the TLR-3 agonist Poly(I:C), a synthetic analog of viral dsRNA, a potent chemokine response is induced, yet this profile is distinct from that triggered through TLR-4.[73] These findings demonstrate that the trophoblast, just like an innate immune cell, can recognize and respond to microbes. As a result, the trophoblast can coordinate an immune response through the recruitment of innate immune cells to the site of infection.

Active Protection of the Trophoblast Against Viral Infection

The trophoblast can not only recognize microorganisms and initiate an immune response; it may also produce anti-microbial peptides and, therefore, actively protect itself against pathogenic organisms. Studies have demonstrated the expression of the antimicrobial human beta defensins 1 and 3 by trophoblast cells.[80] Secretory leukocyte protease inhibitor (SLPI), which is a potent inhibitor of HIV infection[81] and inducer of bacterial lysis,[82] has also been found in trophoblast cells.[83] The expression of TLR-3 by trophoblast cells may explain how the placenta regulates the expression of these antimicrobial factors. Indeed, stimulation of first trimester

trophoblast cells, through TLR-3 with Poly (I:C), promotes the production and secretion of SLPI and beta Interferon (IFN-β), two important anti-viral factors.[84] These factors are the first line of defense against viral infections and have the potential to activate multiple intracellular pathways.[85] Therefore, IFN-β and SLPI production by trophoblast cells in response to a viral infection at the maternal-fetal interface may represent a potential mechanism by which the placenta prevents HIV transmission to the fetus during pregnancy. Together, these observations support the concept that the trophoblast is able to protect itself and the fetus from infectious pathogens.

TLRs and Pregnancy Complications

While the trophoblast has protective properties towards infections and can coordinate the immune system for enhanced responses against microorganisms, infection, nevertheless, is a common mechanism of disease. Intrauterine infections have been associated with cases of preterm labor[30,35,36] and other pregnancy complications, such as intrauterine growth restriction (IUGR) and preeclampsia.[32-34,86] Thus, an infection at the maternal-fetal interface represents a significant threat to both fetal well-being, as well as the success of a pregnancy. While innate immune cells may be important during normal pregnancy for resolving infections at the maternal-fetal interface, these same leukocytes may contribute to the pathology of certain pregnancy complications (Fig. 1). In abnormal pregnancies, such as prematurity or preeclampsia, decidual tissues contain elevated levels of macrophages, neutrophils and NK cells and such leukocyte distributions are altered.[75,87-91] Similarly, in animal models of preterm labor and pregnancy failure, where the delivery of microbial products are used to initiate disease, the decidua becomes infiltrated with these same innate immune cells.[92-94] Such altered immune responses at the maternal-fetal interface may significantly impact the pregnancy.

Toll-like receptors may also function as a link between a dangerous immune response and pregnancy complications, many of which are associated with elevated placental apoptosis.[95-99] Indeed, we have recently demonstrated that TLR-2 ligation by bacterial peptidoglycan directly induces first trimester trophoblast cells to undergo apoptosis, rather than to produce cytokines.[68] In contrast, high levels of LPS acting through TLR-4, triggers first trimester trophoblast cells to produce pro-inflammatory cytokines, including TNFα and IFNγ, which in turn may induce trophoblast cell apoptosis.[100-104] Therefore, while LPS does not directly induce trophoblast cell death, the intense inflammatory response generated by either the trophoblast or decidual immune cells following its activation may provide an alternative mechanism for the induction of trophoblast apoptosis.[105] Thus, we predict that certain intrauterine infections during pregnancy may have either a direct or indirect effect upon trophoblast cell survival, depending upon which TLR is activated. We have also observed that TLR-2 expression in fetal membranes is significantly elevated in women with chorioamnionitis, while TLR-4 expression by interstitial trophoblast cells in increased in patients with preeclampsia.[106,107] Since TLR-4 levels appear to be upregulated by pro-inflammatory cytokines, such as TNFα,[107] altered TLR expression may exacerbate certain pathological mechanisms. Together, all these data suggest that while on one hand TLRs function as important sensors for the trophoblast, allowing it to coordinate the local immune response and promote cell invasion and placental formation; TLRs may also provide the bridge for placental recognition of danger signals and a subsequent shift in the type of response generated may have harmful consequences for the pregnancy.

Summary

Our studies provide an alternative perspective on the role of the maternal innate immune system and its interactions with the trophoblast during pregnancy. We believe that the field of Reproductive Immunology needs to reevaluate its focus, and modify the immunological paradigm of pregnancy from a graft-host interaction towards a symbiotic interaction. As we learn more about the regulation of the expression and function of TLRs during pregnancy we will better understand the cellular cross talk existing at the maternal fetal interface.

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CHAPTER 20

Inherited Thrombophilias and Early Pregnancy Loss

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Introduction

Inherited thrombophilias are a heterogeneous group of conditions which have been associated with a variety of pregnancy complications, including early and late fetal loss, intrauterine growth restriction, abruptio placentae, and preeclampsia.[1] As the functional significance of the burgeoning list of thrombophilic conditions is better understood, more rational and thoughtful approach to their detection and usefulness in clinical practice will likely emerge. While dominant conditions, such as antithrombin deficiency are rarely present without clinical manifestations, other less thrombogenic mutations, such as factor V Leiden, often are not associated with obvious pregnancy complications, as noted by the finding that the presence of heterozygous factor V Leiden is associated with a 0.2% risk of maternal thromboembolism. Emerging data suggests the quality and quantity of thrombophilic conditions, in addition to genetic and environmental influences create a 'threshold milieu' for the clinical manifestation of these heterogeneous prothrombotic conditions. This review will summarize the current knowledge of thrombophilic conditions and their association with first trimester pregnancy outcome.

Pregnancy Related Hemostatic Alterations

It is well accepted that normal pregnancy is a pro-thrombotic state. Haemostatic changes in pregnancy that tend to create a pro-thrombotic milieu have been well documented.[2-10] The circulating levels of several of the coagulation factors including factor fibrinogen, factors VII, VIII, X and von Willebrand factor are increased. Concomitantly, there is a decrease in the natural anticoagulant system, with impressive lower levels of protein S[11] and increased resistance to activated protein C (APC). Impairment of the fibrinolytic process exists, as evidenced by increased levels of plasminogen activator inhibitor -1 and 2 (PAI-1 & PAI-2) along with increased levels of thrombin activatable fibrinolysis inhibitor (TAFI).[12]

Inherited Thrombophilias: Factor V Leiden

Merely a decade ago, the most commonly identified inherited thrombophilias consisted of deficiencies of protein S, C and antithrombin. However, in 1994, Dalhback reported an association of a mutation in the factor V gene and an increased risk for thrombosis.[13] This factor V Leiden (FVL) mutation arises from a (G→A) mutation in nucleotide 1691 of the factor V gene's 10th exon resulting in a substitution of a glutamine for an arginine at position 506 in the factor V polypeptide (FV Q506). The resultant amino acid substitution impairs activated PC/PS's inactivation of factor Va. Functionally, Factor V becomes resistant to cleavage by activated

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protein C.[13] The factor V Leiden mutation is primarily inherited in an autosomal dominant fashion.[14,15] The FVL mutation is common, being present in 5.2% of caucasians, 1.2% of African Americans, and 5-9% of Europeans.[16] It is rare in Asian and African populations.[14,16] It has been reported that heterozygosity for the factor V Leiden mutation is present in 20 to 40% of nonpregnant patients with thromboembolic disease while homozygosity of FVL is associated with a higher (> 100-fold) risk of thromboembolism.[14]

Prothrombin Gene Mutation G2010A

Shortly after the discovery of the FVL mutation, a mutation in the prothrombin gene, prothrombin gene mutation 20210A (PGM) was identified, and was found to be associated with an increased risk of thrombosis,[17] and pregnancy complications.[18] Heterozygosity for a mutation in the promoter of the PGM is not as common as the FVL mutation, being present in 2-3% of Europeans. PGM leads to increased (150-200%) circulating levels of prothrombin and an increased risk of thromboembolism.[14] This mutation has accounted for 17% of thromboembolism in pregnancy in one large study.[18] The estimated risk of thrombosis in an asymptomatic pregnant carrier is 1/200 or 0.5%.[18] Similar to the state of homozygosity of FVL mutation, PGM confers a high risk of thrombosis.[14]

Antithrombin Deficiency

The most thrombogenic of the known inherited thrombophilias is antithrombin deficiency (AT), conferring a 70-90% lifetime risk of thromboembolism.[14,19] Antithrombin inhibits thrombin, and also inactivates factors Xa, IXa, VIIa and plasmin.[14] Antithrombin's anticoagulant effect is potentiated by heparin 5,000 to 40,000-fold. Deficiencies in AT result from numerous point mutations, deletions, and insertions, and are usually inherited in an autosomal dominant fashion.[20] Type I antithrombin deficiency, the most common deficiency is characterized by concomitant reductions in both antigenic protein levels and activity. Type II deficiency is characterized by normal antigenic AT levels but decreased activity. Type II deficiency is further classified by the site of the mutation (e.g., RS- reactive site; HBS- heparin binding site; PE-pleiotropic functional defects).[14,20] Fortunately, the prevalence of AT deficiency is low, 1/1000 to 1/5000. It is only present in 1% of patients with thromboembolism.[19] The risk of thrombosis among affected patients is up to 60% during pregnancy and 33% during the puerperium.[21]

Protein C Deficiency

Protein C (PC) is a vitamin K-dependent 62,000 mol wt glycoprotein substrate that is a precursor to a serine protease, activated protein C (APC).[22] PC is activated to APC by thrombin in the presence of thrombomodulin (TM) on the surface of endothelial cells. Factor Va and factor VIIIa are inactivated by APC, with cofactors PS and factor V. As a result of inactivation of factors Va and VIIIa, thrombin generation is decreased.[22] Deficiencies of PC result from numerous mutations. Two primary types are recognized: Type I, in which both immunoreactive and functionally active PC levels are reduced; and Type II, where immunoreactive levels are normal but activity is reduced.[21] The prevalence of PC deficiency is 0.2-0.5%, and its inheritance is autosomal dominant.[21]

Protein S Deficiency

Protein S (PS) a vitamin K- dependent 69,000 mol wt glycoprotein which has several anticoagulant functions including its activity as a nonenzymatic cofactor to the anticoagulant serine protease APC.[23] PS has a plasma half-life of 42 hr, considerably longer than protein C whose half life is approximately 6-8 hrs. Circulating PS exists in both free (40%) and bound (60%) forms. Plasma PS is reversibly bound (60%) to (C4BP) C4b-binding protein, which serves as a carrier protein for PS. Protein S has also been shown to have an APC-independent anticoagulant function in the direct inhibition of the prothrombinase complex. PS also inhibits thrombin activatable fibrinolysis inhibitor (TAFI).[24]

Protein S deficiency presents with one of three phenotypes: Type I, marked by reduced total and free forms; Type II, characterized by normal free PS levels but reduced APC cofactor activity; and Type III, in which there are normal total but reduced free PS levels. Different mutations have highly variable procoagulant sequelae making it extremely difficult to predict which patients with PC or PS deficiencies will develop thromboembolism. The prevalence of PS deficiency is 0.03-1.3%, and inheritance is autosomal dominant.[25]

Protein S is associated with an increased risk of venous thromboembolism, and has been found in 16% of cases of maternal thromboembolism.[18] Neonatal purpura fulminans and extensive necrosis are rare presentations of homozygous PS deficiency.

Pregnancy is associated with decreased levels of protein S (PS) activity and free PS antigen in the majority of patients.[26] Most normal pregnancies acquire some degree of resistance to activated protein C (APC), when measured by the first generation global assays and tests that measure endogenous thrombin potential.[27,28] Factor X, its activation to FXa and participation in the activation of prothrombin, is a central element in the generation of thrombin.[29] It is possible that derangements in the control of factor Xa in contribute to adverse prothrombotic sequellae in pregnancy.

The significance and degree of the decrease in PS levels commonly seen in pregnancy has not been rigorously evaluated. To address this question, we compared second and third trimester PS levels in 51 healthy women with a normal pregnancy outcome with 51 healthy women with a poor pregnancy outcome. Protein S levels were significantly lower in the second and third trimesters among patients with adverse pregnancy outcome, defined as, intrauterine growth restriction, preeclampsia, preterm delivery, preterm rupture of membranes associated with preterm delivery, and bleeding in pregnancy, compared to patients with normal pregnancy outcome. Protein S levels were significantly lower in the 2nd and 3rd TRI's among patients with APO compared to patients with NPO (2nd TRI 34.4 ± 11.8% vs 38.9 ± 10.3%, $p < 0.05$, respectively; and 3rd TRI 27.5 ± 8.4 vs 31.2 ± 7.4, $p < 0.025$, respectively).[30]

In a follow up study (n = 82 patients in each trimester) free protein S antigen levels ('functional protein S'), were found to be low in all three trimesters [first trimester, 39% (SD 10.5), second trimester 35.6% (SD 8.4), third trimester 27.9 (SD 7.0), compared to the reference range nonpregnant of 88% (SD 19), $p < 0.05$.[31] Derangements of early pregnancy PS decrease are underway.

Protein Z Deficiency

Insights into the mechanism of thrombophilic conditions in pregnancy have become evident through new findings associated a newly described thrombophilic condition, called protein Z deficiency. Protein Z is a 62 kDa vitamin K-dependent plasma protein that serves as a cofactor for a PZ-dependent protease inhibitor (ZPI) of Factor Xa.[32] Protein Z is critical for regulation of factor Xa activity[33] in addition to tissue factor pathway inhibitor.[34-36] Protein Z increases rapidly during the first months of life followed by slow increases during childhood, with adult levels reached during puberty.[37,38] Protein Z deficiency influences the prothrombotic phenotype in factor V Leiden patients,[39] and low plasma PZ levels have been reported in patients with antiphospholipid antibodies.[40,41] There also some evidence suggesting a role of PZ deficiency to a bleeding tendency.[42] While controversy exists surrounding the association between ischemic stroke and PZ deficiency (< 1.0 ug/ml),[43] there is a high prevalence of PZ deficiency in patients with unexplained early fetal loss (10th to 19th weeks).[44]

Gris et al noted that a significant number of patients with PZ deficiency in the early fetal loss group with the level of < 1 μg/ml in 44 of 200 patients, and especially among patients with fetal demise between the beginning of the 10th week and end of the 15th weeks of gestation.

These authors found an increased risk of fetal loss associated with PZ deficiency (odds ratio of 6.7, 95% CI 3.1-14.8, $p < 0.001$), and noted that the patients with late fetal loss and recurrent miscarriages had lower PZ levels.[45]

We found that there was a significant decrease in the PZ levels in patients (n = 51) with a variety of adverse pregnancy outcomes (APO), including IUGR, preeclampsia, preterm

delivery and bleeding in pregnancy compared to women (n = 51) with normal pregnancy outcomes (NPO) (2nd trimester 1.5 ± 0.4 vs 2.0 ± 0.5 μg/ml, $p < 0.0001$; and 3rd trimester 1.6 ± 0.5 vs 1.9 ± 0.5 μg/ml, $p < 0.0002$). Protein Z levels at the 20th percentile (1.30 ug/ml) were associated with an increased risk of adverse pregnancy outcome [Odds ratio 4.25 (1.536-11.759, with a sensitivity of 93%, specificity 32%).[30] In the same group of patients, protein S levels were significantly lower in the 2nd and 3rd TRI's among patients with APO compared to patients with NPO (2nd TRI 34.4 ± 11.8% vs 38.9 ± 10.3%, $p < 0.05$, respectively; and 3rd TRI 27.5 ± 8.4 vs 31.2 ± 7.4, $p < 0.025$, respectively). We theorize that decreased PZ and PS levels are additional risk factors for APO. We found mean 1st TRI PZ level was significantly lower among patients with APO, compared to pregnant controls (1.81 ± 0.7 vs 2.21 ± 0.8 μg/ml, respectively, $p < 0.001$). In patients with other known thrombophilic conditions, those with APO had a tendency for lower mean PZ levels compared to those TP women with NPO (1.5 ± 0.6 vs 2.3 ± 0.9 μg/ml, respectively, $p < 0.0631$).

Gris et al found a inverse correlation between anti-protein Z IgM antibody levels and protein Z concentrations ($p = -0.43$) in patients with recurrent embryonic loss and PZ deficiency.[46] The relationship between PZ antibodies and PZ levels is not straightforward. Anti-protein Z IgG antibody and anti-protein Z IgM antibody levels were not correlated with protein Z levels in the entire cohort of patients with normal and abnormal outcomes. Gris et al has postulated that perhaps anti-protein Z antibodies contribute to enhanced immune complex formation, resulting in lower levels of protein Z. In a cross sectional study with 82 patients in each trimester comparing free protein S antigen, protein Z, IgG and IgM antibodies to PS and PZ,we found that: protein S levels were correlated with PS IgG only in the second trimester (r 0.25, $p < 0.05$); second and third trimester PZ levels correlated with anti-PZ IgM (r 0.26 and -0.28 resp, $p < 0.05$).[31] Overall, PS IgM and PZ IgM in all three trimesters were associated with higher levels compared to a nonpregnant healthy group of adults serving as our reference range, as compared to PS IgG and PZ IgG, which were not significantly elevated in the first trimester. This preliminary data suggest an evolution of immune response to the maternal coagulation system; this immunological response requires further inquiry.

Gris et al carried out a prospective randomized trial comparing the LMWH enoxaparin (40 mg/day) with low-dose aspirin (100 mg/day) in 160 women with one unexplained fetal loss (≥ 10th week of gestation) and either factor V Leiden, prothrombin 20210, or protein S deficiency.[45] Treatments were started at 8 weeks' gestation. The live birth rate was 86% in the enoxaparin-treated women versus 29% in the aspirin-treated group (OR for live birth with LMWH 15.5, 95% CI 7?34). Birth weights were higher and there were fewer small-for-gestational-age infants in the enoxaparin group. Both groups of patients received folic acid 5 mg/day. Gris et al found that the presence of protein Z deficiency or the presence of protein Z antibodies was more frequently present in cases of treatment failures (respectively, $p = 0.20$ and $p = 0.019$) as was the complex of protein Z deficiency positive antiprotein Z antibodies ($p = 0.004$; 15 of the 20 cases led to pregnancy failure, 9 being treated with aspirin and 6 with enoxaparin.

Hyperhomocysteinemia and Methylenetetrahydrofolate Reductase Thermolabile Mutant Gene Mutation (MTHFR C677T)

Homocysteine is normally generated from the metabolism of the amino acid methionine. It normally circulates in the plasma at concentrations of 5-16 μmol/L.[21] Deficiencies in vitamins B6, B12, and folic acid can result in elevated levels of homocysteine in the setting of inherited hyperhomocysteinemia. Homocysteine levels can vary with diet, however, and normal levels in pregnancy are slightly lower than nonpregnant values.

Hyperhomocysteinemia can be diagnosed by measuring fasting homocysteine levels by gas-chromatography-mass spectrometry or other sensitive biochemical means. The disorder is classified into three cateories according the extent of the fasting homocysteine elevation: severe (> 100 μmol/L); moderate (25-100 μmol/L), or mild (16-24 μmol/L). Methionine loading can improve diagnostic sensitivity. Severe hyperhomocysteinemia results from an

autosomal recessive homozygous deficiency in either cystathionine β-synthase (CBS), a prevalence of 1/200,000 or methylenetetrahydrofolate reductase (MTHFR).[21] Clinical manifestations of hyperhomocysteinemia include neurological abnormalities, premature atherosclerosis and recurrent thromboembolism.

The mild and moderate forms can result from autosomal dominant (heterozygote) deficiencies in CBS (0.3-1.4% of population) or from homozygosity for the 667C-T MTHFR thermolabile mutant, present in 11% of white European populations.[21] Patients with mild and moderate hyperhomocysteinemia are at risk for atherosclerosis, thromboembolism, fetal neural tube defects and recurrent abortion.[21] There are conflicting data on the link between hyperhomocysteinemia and recurrent spontaneous abortions abortion.[21,47-49] The predictive value of homocysteine levels in pregnancy has not been particularly useful. An older meta-analysis of the association between hyperhomocysteinemia and pregnancy loss prior to 16 weeks suggested a weak association with an odds ration of 1.4 [1.0-2.0].[50] The natural history of the MTHFR mutation in pregnancy has not been well documented. The recent meta-analysis by Rey concluded that MTHFR was not associated with an increased risk of fetal loss.[51]

Elevated Levels of Type-1 Plasminogen Activator Inhibitor (PAI-1) and Homozygosity for the 4G/4G Mutation in the PAI-1 Gene

Plasminogen activator inhibitors are part of a larger family of serine protease inhibitors, often referred to as serpins (serine protease inhibitors) that have diverse functions, including blood coagulation, fibrinolysis, and cell migration.[52,53] PAI -1 and PAI-2 regulate tissue type and urokinase type plasminogen activators, respectively (t-PA and u-PA). t-PA and u-PA regulate fibrin degradation via the conversion of plasminogen to plasmin, and are also involved in the remodelling of extracellular matrix.[54] PAI-1 and PAI-2 are found in the blood of women with normal pregnancies, and their levels tend to rise with advancing gestation.[55] In preeclamptic patients, the vascular endothelium is responsible for the majority of the elevated PAI-1 plasma levels, with platelets accounting for a smaller proportion.[56] Unlike PAI-1, which is found in a variety of nonpregnant disease states, PAI-2 expression has been identified in a limited number of cells, principally placental trophoblasts, macrophages, and various malignant cell lines.[56,57]

The significance of the elevations in PAI-I activity or antigen or the presence of the 4G/4G PAI-1 mutation is uncertain. It is well known that pregnancy is associated with elevated levels of PAI-1, and higher levels are noted in cases of preeclampsia or intrauterine growth restriction (either during manifestations of the disease process, or shortly prior to their manifestation in the case of preeclampsia).

Homozygosity for the 4G/4G mutation in the type-1 plasminogen activator inhibitor (PAI-1) gene leads to a 3 to 5-fold increased level of circulating PAI-1.[21] The significance of the 4G/4GPAI-1 mutation is uncertain. The contribution is of this prothrombotic mutation to thromboembolic events has been called into question, as evidenced by the recent review by Francis.[59] A large multicenter study did not find a relationship between any of the inherited thrombophilic conditions and fetal loss, but they achieved approximately < 30% power to detect a difference.[60] Polymorphisms of the thrombomodulin gene are associated with an increased risk of thrombosis, and the pregnancy implications are unclear at this time.[61] Interestingly, pregnant patients with thrombophilia and subsequent adverse pregnancy outcomes have been demonstrated to exhibit a decreased first trimester response to thrombomodulin in an activated partial thromboplastin time (APTT) system.[62]

Screening for Inherited Thrombophilia Conditions in Patients with a History of Fetal Loss

Several studies examined thrombophilia and fetal loss (Table 1). In a meta-analysis of 31 studies, Rey et al found that FVL was associated with increased risk of late fetal loss (OR 3.26, 95% CI 1.82-5.83).[63] Gris et al, found a positive correlation between the number of stillbirths

Table 1. Fetal loss and thrombophilic conditions

Author	Year	# Fetal Loss	FVL Pos (%)	PGM Pos (%)	PS Deficiency (%)	References
Preston	1996	141	27%		29%	84
Gris	1999	22	68%	28.6%	92%	64
Dizon-Townson	1997	29	41.4%			85
Kupferminc	1999	12	25%	0		86
Tal	1999	9	22.2%			87
Lindqvist	2000	269	4.5%			88
Martinelli	2000	11	45.4%			65
Kupferminc	2000	23		13.0%		89
Murphy	2000	16	18.8%			90
Alfirevic	2001	7		33.3%	42.9%	91
Many	2002	6	50%			67
Alonso	2002	8	0%	12%		92
Rey	2003	180	15%		77.3%	63
Hefler	2004	94	10.6%	7.4		60

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and the prevalence of thrombophilias among 232 women with previous late fetal loss (22wk) and 464 controls.[64] In the same study, PS deficiency was found in 9 of 84 (10.7%) with at least 2 stillbirths, and the presence of FVL was associated with a high risk of fetal loss > 22 wks (OR 7.83, 95% CI 2.83- 21.67).[64] Martinelli et al found that the risk of late fetal death (> 20wk) was 3x higher if the patient was a carrier of either FVL or PGM mutation.[65] The relative risk of carriers (FVL, PGM) for late fetal loss was 3.2 (1.0-10.9), 3.3 (1.1- 10.3) respectively. Martinelli et al evaluated recurrent late loss and found that FVL was present in 28.6% of patients with recurrent late loss.[66]

There are fewer studies evaluating the rates of PGM and PS deficiency in patients with fetal loss. The prevalence of PGM ranges from 0 to 33%, and for PS deficiency 29-92% (Table 1). Rey et al pooled data from nine studies (n = 2087), and found a significant association between fetal loss and the PGM.[63] The presence of PGM was associated with recurrent fetal loss before 25 wks (n = 690 women), OR 2.56, 95% CI 1.04-6.29) and with nonrecurrent fetal loss after 20 wks, (five studies, n = 1299), OR 2.3, 95% CI 1.09-4.87). The most recent study to evaluate the association between thrombophilic mutations and fetal death (median gestational age 34 wks, with range 20-42 wks), did not find any significant association with FVL, PGM or PS deficiency.[60] In contrast, two other studies that examined recurrent fetal loss and PS deficiency found a significant association (OR 14.7, 95% CI 0.99-218).[64,67] Rey et al found that PS deficiency was associated with nonrecurrent loss after 22 wks in 3 studies (n = 565), OR 7.39, 95% CI 1.28- 42.83).[63]

Early Pregnancy Loss

Early pregnancy loss before 13 weeks occurs in about 10-15% of all pregnancies and recurrent loss affects 1-3% of women of reproductive age.[68] Single spontaneous abortion is quite common and typically is caused by random genetic disorders that do not recur in a subsequent pregnancy.[69]

Recurrent early pregnancy loss may be primary or secondary, and investigation of the etiology of the losses requires a thorough evaluation of personal medical history, inherited and

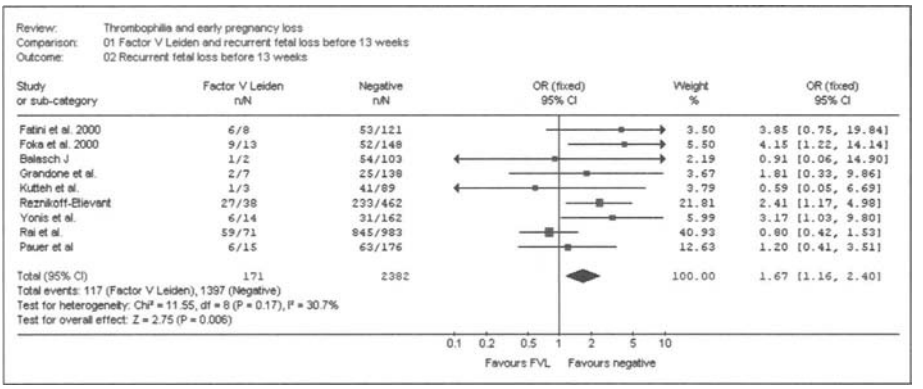

Figure 1. Pregnancy loss and factor V Leiden.

acquired thrombophilic conditions. It is important to differentiate between embryonic and fetal loss to be able to understand the pathological mechanisms responsible for miscarriage, distinguishing the different stages of development and enabling specific causes and processes to be identified.

These ideal considerations, however, has not been generally applied in published studies. In three recent systematic reviews, the diversity among included studies implies that meta-analyses are performed including heterogeneous studies.[63,70,71] In general, it may be concluded that women with combined thrombophilia have a higher risk of fetal loss than women with a single thrombophilic factor. Also, the presense of thrombophilic factors increase the risk of late fetal losses and severe pregnancy complications more than they increase the risk of recurrent first trimester loss.

Among the studies on recurrent first trimester loss patients, the study groups often include women with later losses or stillbirths without separate analyses.[71] But even studies that only included recurrent first trimester losses, or had performed separate analyses on these women, the studies had different cut off limits for gestational age (10, 12 or 13 weeks). Furthermore, entry criteria varied from two or more miscarriages, or three or more miscarriages, and analyses were not carried out considering whether the recurrent losses occurred in women who were never successful in achieving a viable liveborn versus those who had recurrent loss following a successful pregnancy. Control groups also differ between studies especially by obstetric history. Consequently, the results of meta-analyses should be viewed considering the heterogeneity of the studies.

Figure 1 illustrates the results of a meta-analysis of studies considering factor V Leiden and and recurrent first trimester loss. The typical OR is 1.67 (1.16-2.40) and statistically significant. One of the most influential studies in the meta-analysis,[72] however, has different entry criteria from the rest, namely that it only included recurrent pregnancy losses before 10 weeks of pregnancy. The OR in that study alone is 2.41 (1.17-4.98), and when it is not included in the meta-analysis, the OR is somewhat reduced (to 1,57 (0.98-2.51).

Thus, the epidemiological analysis of these studies does not support the hypothesis that first trimester embryos (< 10 weeks) are protected from the adverse effect of thrombophilia.

Figure 2 illustrates the results of a meta-analysis of studies considering prothrombin gene mutation G20210A and spontaneous first trimester loss. The typical OR is 2.25 (1.20-4.21) and statistically significant. In a study of recurrent spontaneous abortions comparing women with thrombophilia with controls, the characteristics of the spontaneous abortions differed between the groups.[73] Thrombophilic women more often had fetal loss (> 10 weeks) whereas controls more often had embryonic losses. In another study[48] the odds for having thrombophilia was actually significantly lower in women with recurrent embryonic losses than in other cases. There are biological considerations that might explain a reduced influence of thrombophilic factors in

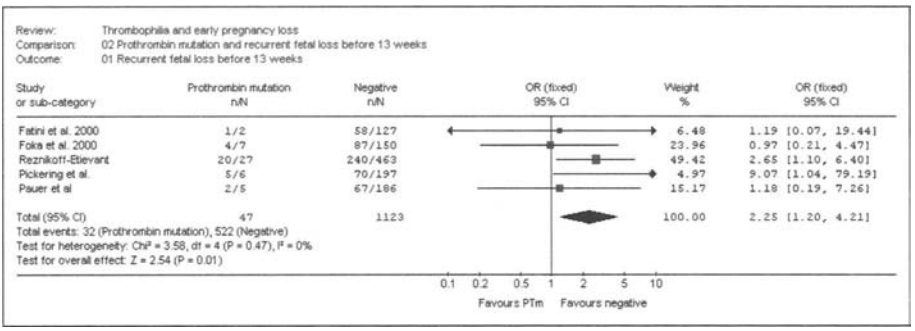

Figure 2. Pregnancy loss and Prothrombin gene mutation G20210A.

very early pregnancy losses (< 10 weeks) - blood flow pattern and oxygenation at the placental site changes at about 10 weeks of gestation. One implication is that thrombophilia exerts a greater influence in pregnancy outcome. However, the current controlled studies find an odds ratio of about 2 in recurrent first trimester losses, which does not differ by gestational age.

In a recent retrospective cohort study of Roque et al, patients with recurrent early pregnancy loss (two or more) were stratified by gestational age into losses before 9 weeks and 6 days, and from 10 weeks to 14 weeks and 0 days.[48] Primary and secondary recurrent losses were not described. Simple correlations between individual factors of thrombophilia and early 1 trimester loss (below 10 weeks) were mostly nonsignificant and negative for all parameters, when compared with those in late first trimester losses. Roque et al investigated the association of thrombophilia (anti-cardiolipin antibodies, lupus anticoagulant, factor V Leiden, prothrombin gene mutation G20210A, homocysteine, deficiencies of protein C, protein S, or antithrombin and one measure of thrombophilia or more). and adverse pregnancy outcome in 491 patients with a history of preeclampsia, IUGR < 10 percentile, fetal loss > 14 wks, recurrent abortion prior to 14 wks, abruption, preterm delivery, and history of thromboembolism. Thrombophilia was associated with an increased risk of fetal loss > 14 weeks, intrauterine growth restriction, abruption and preeclampsia. There was a 'dose dependent' increase in abruptio risk (OR 3.6, 95% CI 1.20-8.6). In those with thrombophilia, preeclampsia was noted in 9%; abruptio in 5.5%; IUGR < 10 percentile in 37%; fetal loss > 14 wks in 16.5%; and preterm delivery in 33%.

Hyperhomocysteinemia does not appear to be associated with recurrent pregnancy loss, as illustrated in Figure 3.

Screening Patients for Thrombophilia

At this time, it advisable to screen for the following inherited thrombophilic conditions, when evaluating patients for adverse pregnancy outcome: FVL, PGM, PS, antithrombin, and PC. At this time, there is insufficient evidence for screening to include PAI-1 levels, PAI-1 4G/5G mutations, MTHFR, homocysteine for the indication of adverse pregnancy outcome. In other circumstances, such as screening for maternal thromboembolism, additional screening may be appropriate.

The following conditions should warrant a thrombophilia evaluation:

i. Personal history of thrombosis (idiopathic, pregnancy, oral contraceptives, trauma, obesity, cancer, underlying medical conditions

ii. 1. History of unexplained loss ≥ 20 weeks
2. History of severe preeclampsia/HELLP
3. History of severe IUGR (< 5 percentile)
4. History of abruption
5. Family history of thrombosis
6. Fetal loss loss ≥ 10 wks

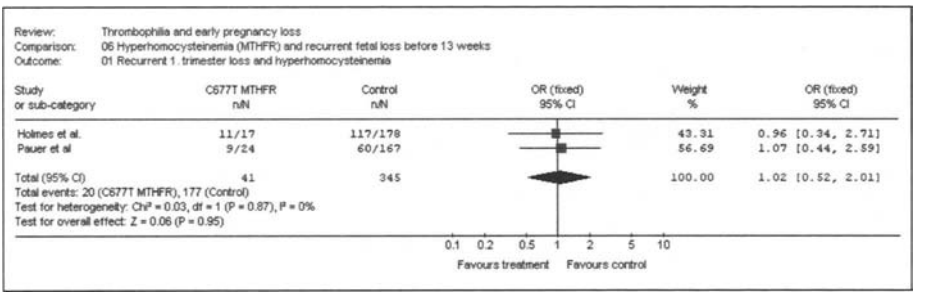

Figure 3. Pregnancy loss and hyperhomocysteinemia.

Laboratory evaluation for the inherited thrombophilias mentioned thus far consists of:

1. Factor V Leiden
2. Prothrombin gene mutation G20210A
3. MTHFR C677T
4. Antithrombin activity
5. Protein C activity
6. Protein S activity, or free protein S antigen
7. Protein Z antigen
8. homocysteine
9. 4G/4G Plasminogen Activator Inhibitor I mutation
10. Plasminogen Activator Inhibitor I activity

Prevention of Adverse Pregnancy Outcome in Patients with Inherited Thrombophilias

Given that an adequate randomized clinical trial has not been performed to assess the risks and benefits of prophylactic antenatal heparin administration, management of patients relies on the results of relatively small studies and expert opinion. Irrespective of thrombophilia status, and according to Duley's systematic review in BMJ it now appears that LDA is associated with a modestly decreased risk of preeclampsia, and fetal loss.[74] Coomarasamy's systematic review also supports the benefits of LDA.[75]

Antenatal Administration of Prophylactic Heparin to Prevent Recurrent Adverse Pregnancy Outcomes in Women with Thrombophilia

Since uteroplacental thrombosis is a common feature of pregnancies complicated by IUGR, severe preeclampsia, abruptio placentae and fetal loss in women with thrombophilia, prophylaxis with heparin has been suggested to prevent their recurrence. The rationale for this approach is that maternal heparin administration will decrease vascular injury and thrombin generation thereby reducing thrombosis in the uteroplacental circulation. There are few published studies describing the use of LMWH with previous adverse pregnancy outcomes.[76-80] Kupferminc et al, treated 33 women with a history of severe preeclampsia, abruptio placenta, IUGR or fetal demise and a known thrombophilia with LMWH and LDA.[79] Treatment was started at 8-12 weeks' gestation and continued until 6 weeks postpartum. Treated patients had a higher birth weight and a higher gestational age at delivery than that of the previous pregnancy. In addition, neither fetal losses nor severe preeclampsia occurred in the treated pregnancies. Riyazi et al reported that treatment with LMWH and low-dose aspirin in patients with previous early-onset preeclampsia and/or severe IUGR and a thrombophilic disorder resulted in a higher birth weight than patients with a comparable history not receiving this

Table 2. Heparin administration to prevent APO

Author/Ref.	Year	# Pts	Drug	Patients Studied	Outcome
Riyazi[76]	1998	26	Nadroparin + ASA 80 mg	Thrombophilia plus prior preeclampsia or IUGR	Treatment associated with lower rates of preeclampsia/ IUGR compared to historical control
Brenner[77]	2000	50	Enoxaparin	Thrombophilia plus recurrent fetal loss	Treatment associated higher live birth (75% vs 20% compared to historical control
Ogueh[78]	2001	24	Unfractionated heparin	Thrombophilia plus IUGR or abruption	No improvement compared to historical control
Kupferminc[79]	2001	33	Enoxaparin + ASA 100 mg	Thrombophilia plus preeclampsia or IUGR	Higher birth weight and gestational age at delivery
Grandone[80]	2002	25	Unfractionated heparin or Enoxaparin	Thrombophilia + APO	Treatment was associated with lower rates of APO (10%) in treated vs (93%) non-treated
Paidas[81]	2004	41	Unfractionated or low molecular weight heparin	FVL or PGM plus history of fetal loss	Treatment was associated with an 80% reduction in fetal loss (OR 0.21, 95% CI 0.11-0.39).
Gris[45]	2004	160	Enoxaparin or 100 mg aspirin; Folic acid 5 mg	Thrombophilia + fetal loss	Enoxaparin was superior to aspirin. 29% patients treated with LDA. 86% treated with Enoxaparin had healthy live birth (OR 15.55, 95% CI, 7-34).

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intervention.[76] The previous studies comprised a small number of patients, had various indications and dosages of heparin therapy and included other medications (Table 2). Hence, adequate conclusions about efficacy and safety of heparin are not possible.

Roque et al investigated the association of thrombophilia and APO in 491 patients with a history of preeclampsia, IUGR < 10 percentile, fetal loss > 14 wks, recurrent abortion prior to 14 wks, abruption, preterm delivery, and history of thromboembolism.[48] Maternal thrombophilia was associated with an increased risk of fetal loss > 14 weeks, IUGR, abruption and preeclampsia. There was a 'dose dependent' increase in abruptio risk (OR 3.6, 95% CI 1.20-8.6). In those with thrombophilia, preeclampsia was noted in 9%; abruptio in 5.5%; IUGR < 10 percentile in 37%; fetal loss > 14 wks in 16.5%; and preterm delivery in 33%.

Paidas et al evaluated a cohort of patients carrying either FVL or PGM who experienced at least one prior APO.[81] A total of 41 patients (28 with FVL, 13 with PGM) had 158 pregnancies. The 41 heparin treated pregnancy outcomes with the remaining 117 untreated pregnancies. Antenatal heparin administration consisted of either enoxaparin, dalteparin or unfractionated heparin. Antenatal heparin administration was associated with an 80% reduction in adverse pregnancy outcome overall (OR 0.21, 95% CI 0.11, 0.39, $p < 0.05$). This relationship persisted if first trimester losses were excluded (n = 111 total pregnancies, OR 0.46 95% CI 0.23, 0.94, $p < 0.05$).

Brenner et al reported on the LIVE-ENOX Study a multicenter prospective randomized trial to evaluate the efficacy and safety of two doses of enoxaparin (40 mg/day or 40 mg bid) in 183 women with recurrent pregnancy loss and thrombophilia.[82] Inclusion criteria were: = 3 losses in the first trimester, = 2 losses in the second trimester, = 1 loss in the third trimester. Compared to the patient's historical rates of live birth and pregnancy complications, enoxaparin increased the rate of live birth (81.4% vs 28.2%, $p < 0.01$ for 40 mg, 76.5% vs 28.3%, $p < 0.01$ for 80 mg), decreased the rate of preeclampsia (3.4% vs 7.1%, $p < 0.01$ for 40 mg; 4.5% vs 15.7%, $p < 0.01$ for 80 mg), and decreased the rate of abruption (4.4% vs 14.1%, $p < 0.01$ for 40 mg; 3.4% vs 9.6%, $p < 0.1$ for 80 mg). There were no instances of maternal bleeding, thrombocytopenia, or neonatal bleeding noted. The lack of a placebo arm, use of historical control, and the small number of patients limit the study conclusions. The study was underpowered to address safety issues with LMWH use.

Gris et al compared administration of low dose aspirin 100 mg/daily with enoxaparin 40 mg daily from the 8th week of gestation in a cohort of patients with a prior loss after 10wks and the presence of heterozygous FVL, prothrombin gene mutation G20210A or protein S deficiency.[45] The authors found that 23/80 patients treated with aspirin and 69/80 patients treated with enoxaparin had a successful pregnancy (OR 15.5, 95% CI 7-34, $p < 0.0001$). Birthweights were higher and there were less small for gestational age infants in the enoxaparin group. Given the limited studies and varied opinion regarding management, a randomized clinical using heparin anticoagulation to prevent adverse pregnancy outcome in the setting of thrombophilia is urgently needed.[81] There are several advantages of low molecular weight heparin over unfractionated heparin, including longer half life, and less heparin induced thrombocytopenia.[83] Prophylactic anticoagulation with low molecular weight heparin consists of enoxaparin 40 mg subq q day or dalteparin 5000U subs q day. For higher risk patients, therapeutic anticoagulation with a weight based regimen, consists of enoxaparin 1 mg/kg q12 hr or dalteparin 200 U/kg q 24 hr.[1]

Conclusion

Inherited thrombophilias are a heterogeneous group of conditions often associated with fetal loss.

Factor V Leiden, Prothrombin gene mutation G2021A, and protein S deficiency are more strongly linked to fetal loss, while other conditions, such as hyperhomocysteinemia, methylenetetrahydrofolate reductase thermolabile mutant gene mutation (C677T), elevated levels of plasminogen activator inhibitor-I or the plasminogen activator inhibitor-I 4G/4G are weakly, if at all, associated with this condition. A correlation between recurrent fetal loss and thrombophilia has been reported in several studies and our meta-analysis of all studies of recurrent first trimester miscarriage provides a typical OR of 1.6 regarding factor V Leiden and a typical OR of regarding prothrombin gene mutation G20210A. Two independent studies indicate that embryonic losses are not affected by thrombophilia, which is compatible with biological considerations. A large epidemiological study, however, find that thrombophilia increases the risk of embryonic loss twofold. Recurrent first trimester loss should be stratified into embryonic (< 10 weeks) and fetal loss (≥ 10 weeks). Hyperhomocysteinemia PS, ATIII are not associated with recurrent early fetal loss. Other thrombophilic conditions, such as protein Z deficiency, appear be associated with an increased risk of pregnancy complications. Antenatal administration of heparin to prevent pregnancy complications has shown promise in small studies, but an adequate randomized, placebo controlled trial is necessary to determine whether heparin administration is beneficial in preventing fetal loss and other adverse pregnancy outcomes.

In future studies on thrombophilic factors and recurrent first trimester fetal loss, it will be important to ensure: that cases are stratified by primary versus secondary abortion, embryonic versus fetal loss and gestational age loss, and that the individual thrombophilic factors are considered both independently and in a multivariate analyses.

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CHAPTER 21

Bi-Directional Cell Trafficking during Pregnancy:
Long-Term Consequences for Human Health

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Abstract

During pregnancy some cells traffic between the fetus and mother and recent studies indicate low levels persist in the respective hosts decades later. Microchimerism (Mc) refers to a small population of cells or DNA harbored by one individual that derive from a genetically distinct individual. Persistent Mc can also arise from cell transfer between twins in utero or after a blood transfusion. Because women are preferentially affected by autoimmune disease, often with an increased incidence in post-reproductive years, fetal Mc has been investigated in diseases such as systemic sclerosis (SSc), autoimmune thyroiditis, primary biliary cirrhosis, Sjögren's syndrome and systemic lupus erythematosus. Maternal Mc has been investigated in SSc, myositis and neonatal lupus. Evidence implicating fetal Mc is strongest in SSc where quantitatively higher levels of fetal Mc have been found and particular human leukocyte antigen (HLA) relationships of mother and child are associated with increased risk of subsequent SSc in the mother. Maternal Mc is implicated in myositis and neonatal lupus. It is unknown how Mc might be involved in autoimmune disease. Mc could play a role in the effector arm of immune responses either directly or indirectly. Microchimeric cells could be targets of an immune response, an intriguing possibility suggested by a recent study in which maternal cells identified in hearts of infants with neonatal lupus congenital heart block were predominantly cardiac myocytes. Alternatively microchimeric cells could be recruited secondarily to diseased tissues and function in tissue repair. The long-term consequences of naturally acquired Mc deriving from pregnancy are not yet known. Because persistent fetal and maternal Mc are not uncommon in healthy individuals it seems likely that beneficial effects may also accrue to the host. Recent advances in this active frontier of scientific research are discussed.

Introduction

The application of molecular techniques to the study of human pregnancy has revealed that bi-directional cell trafficking occurs between the mother and the fetus.[1] The long-term persistence of fetal cells in the mother and maternal cells in her progeny leads to the coexistence of at least two cell populations in a single person.[2,3] Microchimerism (Mc) refers to a small nonhost cell population (or DNA quantity) from one individual harbored by another individual. Other potential sources of Mc can occur as a result of cell trafficking between twins in utero and after a nonirradiated blood transfusion.[4,5] Substantial levels of fetal DNA have been detected in the circulation of women undergoing elective termination and it is presumed that persistent fetal Mc occurs after spontaneous or induced abortion.[6] Theoretically, cells could transfer from an

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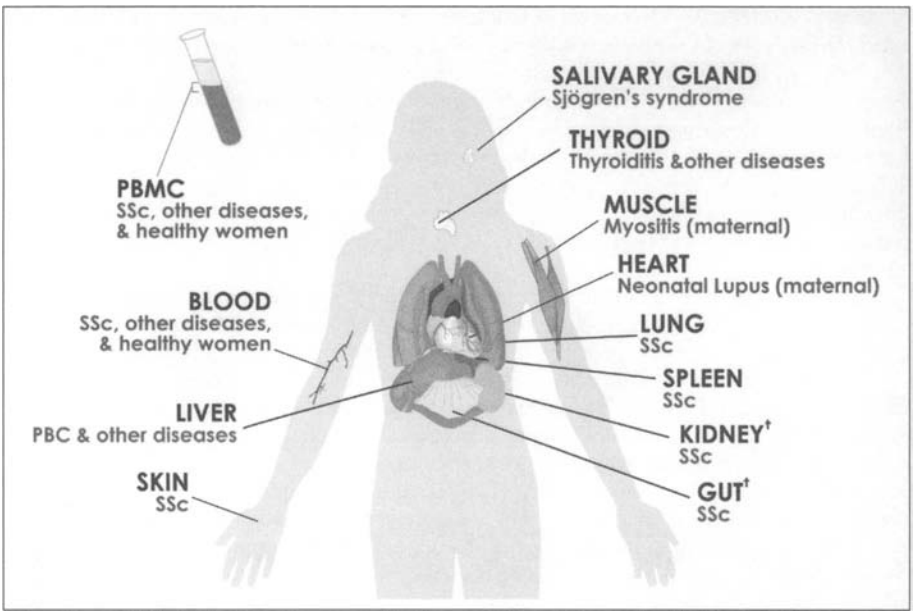

Figure 1. Microchimerism in human health and disease. SSc = systemic sclerosis, PBC = primary biliary cirrhosis, PBMC = peripheral blood mononuclear cells, T = small series of autopsy cases.

older to a younger sibling via the maternal circulation or from sexual intercourse, although these possibilities remain to be investigated.

The concept that naturally acquired Mc from pregnancy might contribute to autoimmune disease arose in part from observations of iatrogenic chimerism in transplantation.[7] After hematopoietic cell transplantation, donor cells may attack the recipient resulting in graft-versus-host-disease (GVHD). Chronic GVHD shares many clinical similarities with autoimmune diseases including SSc (also called scleroderma), primary biliary cirrhosis, Sjögren's syndrome, and sometimes myositis and systemic lupus erythematosus.[8] Other observations contributing to the hypothesis included the predominance of autoimmune diseases in women especially after reproductive years and the observation that the donor-recipient human leukocyte antigen (HLA) relationship is a critical component of both chronic GVHD and graft rejection.[7] The particular HLA genes and the HLA-relationship of host and microchimeric cells likely represent important factors in whether Mc has a detrimental, neutral or possibly even beneficial effect on the host. Although beneficial effects of Mc have not been specifically shown, it seems likely that fetal and maternal Mc provide benefits to the host as both are commonly found in healthy individuals. This chapter reviews recent investigations of fetal and maternal Mc and considers implications of these studies to human health and disease (Fig. 1).

Fetal Mc in SSc

Most studies of fetal Mc test for male DNA or male cells as a marker of fetal Mc in women with sons, although not all studies provide the pregnancy history of study subjects. Initial studies of fetal Mc in autoimmune disease focused on SSc, a disease with clinical similarities to chronic GVHD. The first report described a prospective blinded study of women with SSc and healthy women who had given birth to at least one son.[9] An assay that had previously been standardized for use in prenatal diagnosis was employed to test DNA extracted from whole peripheral blood and provide a quantitative assessment of male DNA. Women with SSc were found to harbor significantly higher levels of male DNA than controls (11 vs. 0.4 mean male

DNA cell equivalents per 16 cc of whole blood respectively). In some women with SSc levels of male DNA were within the highest quartile of fetal Mc observed in women currently pregnant with a normal male fetus, even though the women with SSc had given birth to their sons decades previously. A quantitative approach was used in the initial study investigating fetal Mc in autoimmune disease because an earlier report suggested persistent fetal Mc is frequent among healthy women (positive in 6 of 8 healthy women with sons).[2] When nonquantitative techniques are used only the Mc frequency can be reported i.e., any positive result in cases versus controls. Most, although not all, subsequent studies have been consistent with an increase in levels of fetal Mc without necessarily an increase in frequency when women with SSc are compared to controls.[10-15]

Further characterization of fetal Mc in peripheral blood necessitated development of additional techniques to allow quantitative testing of different blood compartments and isolated cellular subsets. Studies were made possible by the application of real-time quantitative PCR (Q-PCR) methods to the study of fetomaternal cell transfer.[1] In peripheral blood, fetal Mc could be due to circulating cells or to release of breakdown products from disease damaged tissues. Employing real-time Q-PCR for a Y-chromosome specific sequence, increased levels of fetal Mc were found in the cellular component but not in plasma from peripheral blood of women with SSc compared to controls.[10] Of initial studies examining peripheral blood cellular subsets, an earlier study used a nonquantitative technique and described the presence of fetal Mc within T and B lymphocytes, monocytes and natural killer cell populations of women with SSc and healthy women.[11] There was no significant difference of Mc frequency in the different cellular subsets in the women with SSc compared to healthy women. More recently, two studies have employed Q-PCR, examining cellular subsets. One report described a decrease in fetal Mc within maternal CD3-positive T lymphocytes including CD8+ cells but noted the majority of patients were taking immune suppressive medication.[10] Another report described an increase of fetal Mc within CD4-positive T lymphocytes in the diffuse form of SSc but did not provide medication use.[12] Further studies of immunologically active peripheral blood subsets are needed—ideally studies of patients who are early in the disease course, including evaluation of immunosuppressive medication use and categorization according to clinical disease characteristics. In addition, other variables will need to be considered that have not yet been established for healthy women, as this is a new investigative frontier. For example, does length of time from pregnancy, number of pregnancies, or type of pregnancy outcome (spontaneous or induced abortion, preeclampsia, preterm birth, etc.) affect fetal Mc levels and/or cellular subset distribution in the mother?

Lesional skin of women with SSc has been tested for male DNA in two studies both with positive results.[16,17] The reports differed in that the former used a nonquantitative technique and described an increased frequency[16] whereas the latter employed a quantitative technique and reported no difference in frequency but did find quantitatively significant differences in women with SSc compared to controls.[17] In the latter study, the male DNA cell equivalents were 4.6 compared to 1.8 in 80ng of tissue in cases and controls respectively. In another study, autopsy tissues from a limited number of women with SSc and controls who had given birth to sons were studied for male cells by fluorescence in situ hybridization (FISH) for Y and X-chromosome specific markers.[18] Male cells were identified in lung, kidney, skin (SSc disease sites), liver, adrenal gland and lymph node of some patients with particularly high numbers in the spleen. High levels of male cells in the spleen were interest in light of a previous study reporting marked splenomegaly in a murine model of Mc in SSc.[19] Mice were treated with vinyl chloride, an agent associated with SSc; significantly increased levels of fetal Mc were observed and correlated with splenomegaly and dermal fibrosis.

The hypothesis linking fetal Mc to autoimmune disease incorporated the proposal that particular HLA genes and the HLA-relationships between host and microchimeric cell populations is likely a key determinant of the effect of Mc on the host. To investigate this aspect of the hypothesis, women and all their children were studied for the HLA class II genes DRB1 (encoding HLA-DR), DQA1 and DQB1 (encoding HLA-DQ). An increased risk of subsequent

SSc in the mother was observed when a previously born child was not distinguishable from the mother's perspective for genes encoding the HLA-DR molecule.[9] Another study purported to show compatibility of either a patient's mother or child as a risk factor for SSc, but unfortunately was not interpretable due to a number of methodological problems including use of controls with an HLA-associated disease and comparison across different HLA-gene families that are not independent of each other.[20] A subsequent study examined HLA-compatibility of the patient's mother in studies of men with SSc and found no difference compared to healthy men.[21]

Additional studies are needed with serial testing of fetal Mc levels and comparison to other inflammatory autoimmune and non-autoimmune diseases. Overall studies in SSc may be summarized as generally showing a significant difference in the quantity of fetal Mc in blood and tissues compared to controls without necessarily showing an increased frequency of fetal Mc. Results available to date lend support to the importance of HLA genes as likely key determinants of whether long-term persistent fetal Mc has the potential for adverse consequences to the host.

How Might Fetal Mc Contribute to Disease Pathogenesis in SSc?

Whether Mc contributes to the pathogenesis of SSc and what mechanisms may be involved are unknown. Differences in Mc between women with SSc and controls could be interpreted as a secondary event in the pathogenic process. Increased risk of subsequent SSc in women who previously gave birth to an HLA-DRB1 compatible child, however, argues against this interpretation. A number of possibilities may be considered. Fetal microchimeric cells could potentially function as effectors or targets of an immune response. Other investigators have described T cell clones, presumably of fetal origin, which react specifically with maternal (mismatched) HLA antigens, expressing a pattern of cytokine production consistent with T-helper type 2.[22] However, caution should accompany direct analogy with mechanisms involved in chronic GVHD where circulating cells are essentially completely replaced by donor cells as levels of naturally acquired fetal Mc are very low (generally far less than 1% of circulating cells). The effect of a small number of cells could be amplified, for example, by presentation of fetal peptides by one host cell to other another host cell, analogous to the "indirect" pathway of recognition, thought to play a role in chronic rejection of organ grafts. A small number of microchimeric cells could potentially dysregulate host-to-host cell interactions in a paracrine fashion through cytokine secretion. The possibilities are not mutually exclusive as more than one mechanism could be contributory. With respect to the contribution of HLA genes, an excess HLA similarity of fetal cells to the mother without complete HLA-identity could hamper the recognition of cells as foreign while promoting autoimmunity by simultaneous presentation of peptides derived from HLA that are similar and dissimilar to self. Thus, Mc could have adverse, neutral (or beneficial) effects on the host, depending upon particular HLA genes and the HLA-relationship between the different cell populations.

Fetal Mc in Autoimmune Thyroid Disease

A disproportionate incidence of autoimmune thyroid disease in women and frequent onset postpartum prompted investigation of fetal Mc.[23,24] In a study of thyroid tissues, the frequency of male DNA was greater in Hashimoto's disease compared to nodular goiter and positive results correlated with women who had sons.[25] Male DNA was likewise more frequent in thyroid tissues from women with Graves' disease compared to controls with adenoma.[26] Employing FISH to identify male cells in thyroidectomy and autopsy specimens from women with multiple thyroid disorders, male cells were found in thyroids from more than half of women with a thyroid disease compared to none in autopsy controls.[27] Women with Hashimoto's thyroiditis harbored male cells at a somewhat greater frequency than women with other thyroid diseases, however male cells were almost as frequent in some non-autoimmune conditions. Suggesting Mc could contribute to tissue repair, one tissue section had male cells that were

indistinguishable from differentiated thyroid follicles. The concept that fetal Mc may be recruited to the thyroid during immunologic injury is supported by observations from a murine model of experimental autoimmune thyroiditis (EAT).[28] Fetal T cells and dendritic cells accumulated within the thyroids of mice with EAT during pregnancy and the early postpartum period. After mating females to males with green fluorescent protein expression, offspring cells expressing the protein could be found in thyroid glands from experimentally immunized pregnant and postpartum mice and not in control nonimmunized mice.

Fetal Mc in Other Autoimmune Diseases

Studies of fetal Mc in other autoimmune diseases have examined Sjögren's syndrome (SS), systemic lupus erythematosus (SLE), and primary biliary cirrhosis (PBC). Direct comparison of study results within a particular disease is often limited by differences in techniques for measuring Mc and in study design (discussed later). In contrast to SSc and thyroid diseases where studies generally lend support to a potential role for fetal Mc, in SS and SLE results are conflicting and in PBC mostly negative. Earlier studies of SS reported no significant difference of fetal Mc in peripheral blood samples between women with SS and controls.[29] Subsequent studies examined DNA extracted from salivary glands with differing results. Nonquantitative methods were used in all studies. One report described an increased frequency of male DNA in minor salivary gland biopsies from women with SS compared to controls (55% vs. 13%).[30] Another study testing for male DNA in labial salivary glands found none in primary SS, but positive results were found in a small number of women with SSc and with SSc and secondary SSc.[31] A third study reported male DNA more often in labial salivary glands of SS patients than controls (36% vs. 0%) and also found positive results in some bronchoalveoloar lavage specimens from a limited number of women with SS.[32] Modest sample sizes were assessed in these studies and future studies of larger numbers of subjects in different clinical disease subsets using quantitative techniques are needed.

In one series of SLE patients, when nonquantitative methods were used no significant difference of fetal Mc was observed in women with SLE compared to controls; however when real-time Q-PCR methods were employed, SLE patients differed significantly from controls.[33] In another series of similar size also employing real-time Q-PCR no difference of frequency or quantity was observed.[34] The latter study, although finding no correlation with disease activity, did report a higher mean fetal Mc level in patients with a history of lupus nephritis. A case report of a woman who died from SLE described male cells in multiple disease-affected tissues including kidney, lung, heart, skin and intestine.[35] SLE has extensive and varied clinical manifestations so that additional studies of large populations of well-characterized patients will be necessary to determine whether fetal Mc plays an role in this disease. From an experimental perspective, rationale exists for exploring a possible role of maternal Mc in SLE since a murine model of SLE is created by the infusion of parental cells into the F1 progeny.[36]

PBC is a chronic, progressive autoimmune liver disease that has features similar to chronic GVHD of the liver. An initial study of PBC found male DNA in the majority of livers of women with PBC but also in the majority of livers affected by other diseases, without a difference of frequency, although the quantity was somewhat greater in PBC.[37] Most studies have reported no significant difference in fetal Mc of women with PBC when compared to other liver disorders.[38-41] The frequency of positive results when testing for male DNA varied over a wide range (18% to 70%), but may be explained at least in part by differences in patient selection as some studies were limited to women with sons while others were not. Although no overall association was observed, another study found fetal Mc more often in PBC patients who had anti-centromere antibodies, an antibody that is associated with the CREST variant of SSc.[39] Overall evidence is lacking to support a causal role for fetal Mc in PBC. However, whether immunologic consequences of fetal Mc might differ depending upon pregnancy type e.g., spontaneous or induced abortion, or whether the total number of sexual partners

correlates with MC has not been investigated in any autoimmune disease. This gap in knowledge is especially evident in studies of PBC since a recent report described an increasing risk of PBC with increasing gravidity with double the percentage of PBC patients reporting five or more children compared to controls.[42]

Maternal Mc in Autoimmune Disease

The transfer of maternal cells into newborn infants has been appreciated at least since the 1960s when routine karyotyping of male infants demonstrated sex chromosome mosaicism.[43] Maternal transfer of erythrocytes, leukocytes, and platelets into the infant's circulation was demonstrated when maternal blood labeled with a tracer was found in the newborn infant's cord blood.[44,45] Although studies of fetal Mc can employ a single PCR assay for male DNA in women with sons, no similar simple approach has been available to study maternal Mc. The FISH technique can be used to visually identify and quantify female cells in a male but is laborious and time intensive. An alternative approach is to target a noninherited nonshared genetic polymorphism. Using this approach, the estimated frequency of maternal cells in cord blood was 42%.[46] Using FISH maternal cells have been detected in multiple newborn tissues including liver, spleen, thymus, thyroid, and skin.[47]

Maternal Mc is implicated in myositis. The frequency of maternal Mc was significantly increased both in peripheral blood mononuclear cells and in muscle biopsies of children with juvenile dermatomyositis as compared to unrelated controls and to unaffected siblings.[48] Inclusion of unaffected siblings as a control group is a study design strength as environmental, and to some extent genetic background are similar. Although not limited to autoimmune myositis, other studies of children with idiopathic inflammatory myopathy also reported an increased frequency of maternal Mc in peripheral blood and in muscle biopsies.[49] The phenotype of maternal cells in muscle tissues was not determined in these studies.

In an early study of SSc maternal-specific DNA was detected using a nonquantitative technique in more than half of all study subjects without a significant difference in patients and controls.[3] A more recent report described an approach in which a panel of HLA-specific real-time Q-PCR assays was developed targeting noninherited nonshared maternal-specific HLA sequences.[50] Employing the panel of quantitative assays and studying a larger number of subjects, maternal Mc was significantly more frequent among SSc patients than healthy women (72% vs. 22%). Quantitative differences were not significantly different in the two groups. As described previously, HLA-compatibility of the mother has also been investigated in men with SSc. Mothers of men with SSc were not compatible more often than mothers of healthy men.[21]

How Might Maternal Mc Contribute to Disease Pathogenesis?

Whether maternal Mc is involved with disease pathogenesis and any mechanism of involvement is not known. However, an intriguing possibility is suggested by a recent report that investigated maternal Mc in neonatal lupus erythematosus syndrome.[51] A technique was developed by which the same cells in a tissue section could be evaluated for phenotype and for sex with concomitant immunohistochemistry and FISH for X- and Y-chromosomes. Autopsy samples from male infants were examined and maternal cells identified in the atrioventricular node and myocardium (up to 2% of all cells in some sections). Interestingly, the majority of maternal cells lacked hematopoietic cell markers instead expressing markers of cardiac myocytes. This finding suggests that microchimeric cells can transdifferentiate, which is consistent with many other recent reports of unexpected cellular plasticity in cells thought to have undergone terminal differentiation (i.e., neurons, hepatocytes).[52] Thus, microchimeric cells could be the target of a host immune response with subsequent fibrosis of the conduction system and eventual heart block. Alternatively, transdifferentiation of maternal microchimeric cells could contribute to tissue repair. The two possibilities cannot be distinguished in this study.

Technical and Study Design Considerations

In studies of Mc, experimental design and techniques are of paramount importance in drawing valid conclusions, because it is a low frequency event and occurs frequently in healthy individuals. Interpretation of studies employing PCR for detection of microchimeric DNA requires knowledge of the sensitivity and specificity of the particular assay employed in addition to specific methodological information such as the number of DNA aliquots tested and number of times testing was conducted. PCR assay specificity may be compromised by cross-reactivity of some Y-chromosome sequences with autosomal sequences yielding false-positive results. Nested PCR techniques are nonquantitative and subject to greater risk of contamination than closed PCR systems (e.g., kinetic PCR). Despite good sensitivity of assays targeting Y-chromosome multi-copy sequences, some Y-sequences vary in copy number between individual men so that only multi-copy Y-chromosome sequences that have a stable copy number between men should be used for quantitative purposes.

Additional sources of variation arise depending upon the compartment of blood being investigated and specific technique used during blood processing. For example, during pregnancy fetal DNA is much higher in maternal plasma than PBMC.[53] In contrast, long-term persistent Mc was essentially limited to the cellular component (and not plasma) in a study of women with SSc and healthy women.[10] The speed of centrifugation and presence of a filtration step during blood processing alters the total DNA detected in the sample and may affect results of testing for Mc.[54] DNA extracted from paraffin-embedded tissues could be subject to contamination because paraffin baths are not routinely changed between samples. Other technological issues arise when studying tissues by FISH as overlapping cells can produce artifacts; only cells with two signals in a well-defined nucleus should be counted.

The preferable study design for investigation of Mc is prospective with inclusion of pregnancy history and other potential sources of Mc. Some studies assayed for male DNA in a female host and reported results as fetal Mc but lacked pregnancy history. Sources of male DNA in a woman to be considered include a prior pregnancy, twin, or blood transfusion, with other potential sources from anolder brother passed via the maternal circulation or possibly sometimes from sexual intercourse. Testing using assays to identify unique genetic polymorphisms will be especially useful in future studies, for example to support fetal origin of male DNA. Finally, disease stage and medications, notably immunologically-active medications at the time of blood draw, should be queried as either could confound a potential association between Mc and disease.

Summary

Bi-directional cell traffic occurs between a mother and child during pregnancy, and Mc persists in respective hosts decades later. Recent studies examining fetal and maternal Mc provide support for the possibility that Mc could contribute to the pathogenesis of selected diseases, particularly SSc, autoimmune thyroid disease, myositis and neonatal lupus. Microchimeric cells could also benefit the host through tissue repair. It is likely that Mc can have adverse, neutral, or beneficial effects on the host, depending upon other factors with HLA genes and the HLA-relationship among cells probably of key importance. Elucidating the mechanisms by which naturally acquired Mc is permitted without detriment to the host may lead to novel strategies with application to prevention and/or treatment of autoimmune diseases. Further investigations in this area of research may also be of benefit in advancing understanding of iatrogenic chimerism, notably in organ and stem cell transplantation.

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CHAPTER 22

Term and Preterm Parturition

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Introduction

Parturition is the act of giving birth.[1] It is a complex process that includes the anatomical, physiological, biochemical and immunological events taking place in the mother, placenta and fetus. These events are responsible for: (1) the preparation of the uterus for labor; (2) labor per se, leading to the delivery of the fetus and placenta; and (3) post-partum uterine involution, breast feeding and adaptation of maternal behavior.

The mechanisms responsible for labor resemble those involved in implantation since both have been likened to carefully regulated inflammatory processes. Indeed, many of the molecular events implicated in parturition have also been associated with the process of implantation.

Abnormal parturition is a major cause of maternal and perinatal morbidity and mortality. Untimely onset of labor increases the risk of perinatal death. Failure to initiate labor or prolonged pregnancy is associated with an increased risk of fetal death.[2] Preterm labor and delivery is the leading cause of perinatal morbidity and mortality worldwide.[3] Mothers also pay a price for labor abnormalities; failure to progress in labor at term (often referred to as dystocia) is the most frequent indication for cesarean section.[4] Intrapartum infection (clinical chorioamnionitis) is a common complication that may result in maternal sepsis.[5] Inadequate hemostasis after delivery of the placenta (i.e., post-partum hemorrhage) is a leading cause of maternal death worldwide.[6] Hence, the importance of labor as a clinical entity. Yet, fundamental questions about the physiology and pathology of human parturition, a process that has played a key role in shaping the evolution of our species, remain unanswered at the beginning of the 21st century. The emphasis of this chapter will be the preterm labor syndrome, given that preterm birth has become the major challenge to modern obstetrics in areas of the world where the problem of maternal mortality has been conquered.

Normal Duration of Pregnancy

The mean duration of human pregnancy is 280 post-menstrual days or 40 weeks in women with a regular 28-day menstrual cycle (adjustments must be made for shorter or longer menstrual cycles). Studying 4000 women with reliable dates and regular menstrual cycles, Kloosterman found that 4.5% of patients delivered on the expected date of confinement (also known as EDC, which corresponds to the Expected Date of Delivery calculated from the Nägele rule), 29% at 40 weeks ± 3 days, and 80% between 38-42 weeks of gestation.[7] Preterm delivery is one that occurs before 37 completed weeks. Post-term pregnancy is one in which the gestation goes beyond 42 weeks.

The duration of gestation varies remarkably among animal species.[8] A combination of fetal genetic and environmental factors appears to play a role, although the precise mechanisms are unknown. Conceptually, the duration of pregnancy should be linked to the time required for

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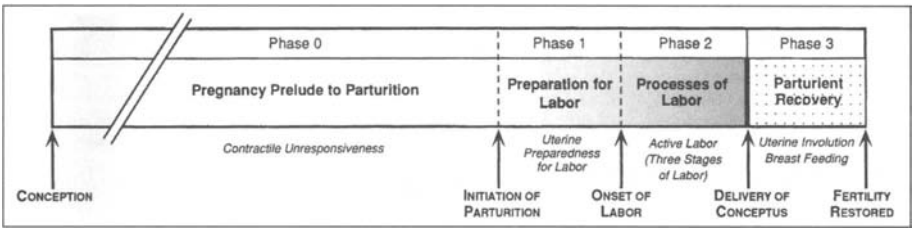

Figure 1. The initiation of parturition and the onset of labor. Reprinted with permission from Cunningham et al.[10]

the birth of a developmentally mature fetus capable of surviving extra-uterine life and for the adaptations to be in place to react to changes in environmental conditions. Thus, mechanisms must exist to synchronize fetal growth and maturation with the maternal process of parturition. Evidence of a gene-environment interaction to determine the length of gestation derives from cross-breeding experiments. Allen et al transferred Thoroughbred embryos to the uteri of smaller Pony mares and Pony embryos to Thoroughbred mares to determine the effect of maternal size on placental and fetal size. These experiments also afforded an opportunity to examine the gene-environment interaction on the duration of gestation. A Thoroughbred in Thoroughbred group and a Pony in Pony group, established by artificial insemination, served as the control groups. The mean duration of gestation of the Thoroughbred in Thoroughbred pregnancy was 339 ± 3 days while the Pony in Pony pregnancy was 325 ± 3 days. The duration of gestation in the experimental groups fell between those two. The Thoroughbred in Pony (also called deprived in utero environment) lasted 332 days ± 2.8 days, while the Pony in Thoroughbred (also called luxurious in utero environment) lasted 331 days ± 2.7 days. The authors concluded that the fetal and maternal genomes had an effect on the duration of pregnancy. A longer gestation was associated with a Thoroughbred genome, whether it was a mare or a foal. The longer gestation in the "luxurious in utero environment" provided by the Thoroughbred mother gestating a Pony suggests that environmental factors operate to determine the duration of pregnancy. Otherwise, the fetus could have been born at the expected gestational age of the Pony. In contrast, the effect of the fetal genome is demonstrated in the Thoroughbred in Pony group in which gestational length was longer than that expected from the maternal genome alone in spite of a "deprived in utero environment." In conclusion, gestational length is likely a function of both genetic and environmental factors.[9]

An Overview of Parturition and Labor

The terms labor and parturition are often used interchangeably, although they are not synonymous. Labor is the acute process by which the fetus is expelled from the uterus, usually within 24 hours. Clinically, labor is defined as a state in which there is increased frequency and intensity of uterine contractions, leading to progressive cervical effacement and dilatation and delivery. In contrast, parturition describes the changes that occur in preparation for, during as well as after labor. The parturitional process lasts weeks while labor lasts hours.

An integrated view of the relationship among implantation, pregnancy, labor and parturition was proposed by Paul MacDonald.[10] He proposed that this relationship could be represented to include four phases, which will be described below (Fig. 1).[10] We have slightly modified slightly this insightful conceptual framework to take into account evidence gathered after MacDonald's initial proposal.

Phase 0 is characterized by myometrial tranquility, a state of cervical unripeness and intact membranes. A key feature of this phase of gestation is that the inherent propensity of the myometrium to contract is harnessed so that the uterine smooth muscle is refractory to natural

stimulants such as oxytocin.[10] The chorioamniotic membranes remain intact. MacDonald proposed that this physiologic state existed for 95% of pregnancy.[10]

Phase 1 of parturition is also called "preparation for labor" and is characterized by suspension of uterine tranquility and the beginning of myometrial responsiveness to agents that stimulate contractility (uterotonic agents). The initiation of parturition (not labor) is considered to be the switch from Phase 0 to Phase 1 and is sub-clinical in nature. There is no current test that can identify this hypothetical switch with certainty. The traditional view has been that there is a progressive nature to the transition from Phase 0 to Phase 1, but this needs to be reconsidered based on clinical and experimental observations, including that parturition could have a reversible and an irreversible course. The irreversible course takes place when the switch from Phase 0 to Phase 1 is followed by Phase 2. However, some maternal insults, such as pyelonephritis, can lead to increased uterine contractility and induce cervical changes.[11,12] Yet, early treatment of pyelonephritis can prevent preterm delivery.[12] Similarly, the administration of antiprogestins can induce a switch from Phase 0 to Phase 1 of parturition, but this is not always followed by progression to delivery (Phase 2).[13,14] Moreover, the administration of 17-alpha hydroxyprogesterone or progesterone may prevent the transition from Phase 0 to Phase 1.[15-17]

Phase 2 corresponds to the process of labor and is clinically characterized by a dramatic increase in the intensity and frequency of uterine contractions, which become painful, progressive cervical effacement and dilatation, and membrane rupture. The term, "onset of labor," specifically refers to the transition from Phase 1 to Phase 2. In 1986, Challis and Lye introduced the concept of "activation" to refer to the changes in uterine function, which take place between Phase 1 and Phase 2 of parturition.[18] From a clinical point of view, some of the changes in Phase 2 can be objectively described with partograms, which display the relationship between cervical dilatation and the station of the presenting part as a function of time. The end of Phase 2 corresponds to delivery of the conceptus. Clinicians often refer to the first stage of labor as the events taking place between the onset of clinical labor and complete dilatation of the cervix. The second stage is the period between full dilatation and delivery of the fetus. The third stage is the interval between delivery of the fetus and expulsion of the placenta. The first stage of labor is clinically important because most labor disorders are diagnosed at this stage (i.e., dysfunctional labor, dystocia, failure to progress in labor, etc.).

Phase 3 refers to the events that take place after delivery and are aimed at the maternal recovery from childbirth, maternal contribution to survival of the infant (i.e., breastfeeding) and restoration of the nongestational state.

Challis and Gibb have proposed that a different set of biochemical mediators are involved in the four phases of parturition.[19] Candidate molecules are displayed in Figure 2.[19] For example, Phase 0 would be mediated by inhibitors of uterine contractility such as progesterone, prostacyclin, relaxin, and parathyroid hormone-related peptide (PTHrP).[19] Phase 1 is considered an active process involving fetal development and maturation. Specifically, the fetal-hypothalamic-pituitary adrenal (HPA) axis is hypothesized to increase the output of cortisol, which ultimately leads to upregulation of a group of genes that encode for contraction-associated proteins, including connexin-43 (the major protein of myometrial gap junctions) and oxytocin receptors, among others.[19] The role of the fetus and the placenta in changing the concentrations of estrogen (uterotropin) and progesterone are considered central to this phase. Phase 2 of parturition refers to stimulation of a "prepared" uterus by uterotonic agents, such as prostaglandins and oxytocin.[19] The latter hormone is also involved in uterine contractility after delivery of the placenta, breast milk ejection and maternal-fetal bonding.

The current descriptions of the mediators involved represent the contribution of many investigators using hypothesis-driven research strategies. The utilization of discovery techniques, such as genomics, proteomics and metabolomics, which allow a comprehensive description of the global changes in the transcriptome, proteome and metabolome, are likely to yield new insights into the process of normal and abnormal parturition and allow integration of much of the research conducted to date.

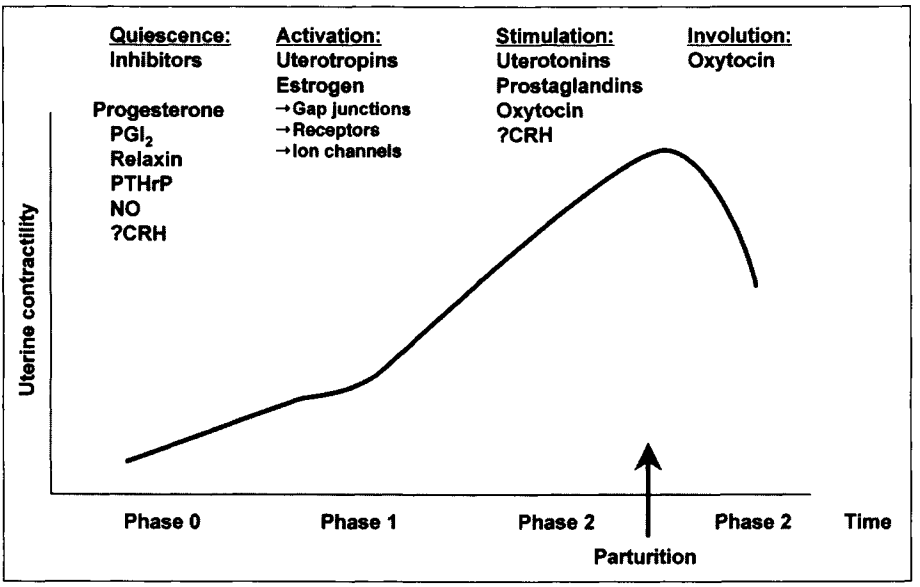

Figure 2. Candidate mediators involved in the four phases of parturition. Reprinted with permission from Challis JRG, Gibb W.[19]

In broad terms labor, may begin because of: (1) removal of inhibitory actions of factors which promote uterine tranquility and a state of cervical unripeness during most of gestation; (2) the effect of stimulatory agents on the same target organs; or (3) a combination of both.

The Common Pathway of Parturition: Components

The common pathway of human parturition has been defined as the anatomic, biochemical, physiologic and clinical events that occur in the mother and/or fetus in both term and preterm labor. The intrauterine components of this pathway include increased uterine contractility, cervical ripening (dilatation and effacement) and decidual/fetal membrane activation (Fig. 3). In addition, systemic processes (endocrinological, metabolic, etc.) are also part of the pathway. For example, term and preterm parturition are associated with an increase in the plasma levels of corticotrophin-releasing hormone (CRH) and cortisol, as well as an increase in caloric metabolic expenditures.[20-29]

Increased Uterine Contractility

Although myometrial activity occurs throughout pregnancy, labor is characterized by a dramatic change in the pattern of uterine contractility that evolves from "contractures" to "contractions." Nathanielsz and colleagues[30,31] defined contractures as epochs of myometrial activity lasting several minutes that are associated with a modest increase in intrauterine pressure and very fragmented bursts of electrical activity on the electromyogram. In contrast, contractions are epochs of myometrial activity of short duration, associated with dramatic increases in intrauterine pressure and electromyographic activity. The switch from a contracture pattern to a contraction pattern occurs during normal labor[32] or it can be induced by pathological events such as food withdrawal, infection and maternal intra-abdominal surgery.[33-35] The switch from a contracture to a contraction pattern is a potentially reversible phenomenon.[33,36-38] The increased birth rate observed immediately after a period of fasting suggests that a fasting-induced switch operates in humans.[37,38]

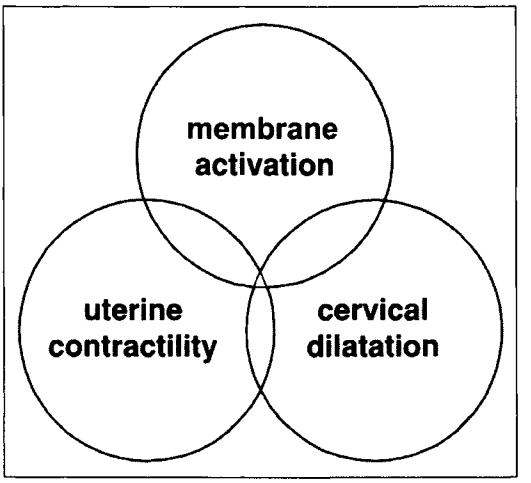

Figure 3. The common terminal pathway of preterm and term parturition. From Romero et al. The Preterm Labor Syndrome. In: Preterm Labor. Elder, Romero, Lamont, eds. Churchill Livingstone, Inc.

The change from the contracture to the contraction pattern begins at night, and labor is preceded by a progressive nocturnal increase in uterine activity of the contraction pattern.[39,40] The circadian nature of this rhythm suggests that uterine activity is under neural control. Plasma concentrations of oxytocin follow a circadian pattern similar to that of uterine contractility,[39] and oxytocin antagonists blunt the nocturnal increase in uterine activity that precedes the onset of parturition in monkeys,[39-43] suggesting that oxytocin may mediate the circadian rhythm in uterine contractility. Oxytocin is produced by human decidua[44-50] and paraventricular nuclei of the hypothalamus, while oxytocin receptors are present in myometrial cells and decidua. This hormone, therefore, may play both an endocrine and a paracrine role in the control of parturition.[45,51-58]

Labor is characterized by a coordinated and effective generation of mechanical forces by the myometrium, which eventually lead to the expulsion of the conceptus. Increased cell-to-cell communication is thought to be responsible for the effectiveness of myometrial contractility during labor. Gap junctions develop in the myometrium just prior to labor and disappear shortly after delivery.[59-63] Gap junction formation and the expression of the gap junction protein connexin 43 in human myometrium is similar in term and preterm labor.[64-68] These findings suggest that the appearance of gap junctions and increased expression of connexin 43 may be part of the underlying molecular and cellular events responsible for the switch from contractures to contractions prior to the onset of parturition. Estrogen, progesterone and prostaglandins have been implicated in the regulation of gap junction formation, as well as in influencing the expression of connexin 43.[69-71] Lye et al[68,72,73] have referred to a set of distinct proteins called "contraction-associated proteins" that are characteristic of this phase of parturition.

Recently, Lye et al proposed that the myometrium undergoes sequential phenotypic remodeling during pregnancy.[74] Specifically, that rat myometrium undergoes changes in the extracellular matrix and contractile proteins which can be grouped in three phenotypes including: (1) Proliferative, when the number of myocytes increase as demonstrated by a higher PCNA cell labeling and protein expression in early pregnancy. This phenotype coincides with a higher myometrial expression of anti-apoptotic proteins (BCl-2 and BCl-xL); (2) Synthetic, when the myometrial cells undergo hypertrophy as demonstrated by a higher protein/DNA ratio in the second half of pregnancy, and coincides with a higher secretion of ECM from the myocytes, in particular collagen I and collagen III, as well as with a high concentration of caldesmin (a

marker of synthetic phenotype); and, (3) Contractile, which occurs at the end of pregnancy and coincides with a low myometrial expression of interstitial matrix and high expression of components of the basement membrane (laminin and collagen IV). In addition, the authors reported a high protein expression of alpha actin in the myometrium in early pregnancy and a high protein expression in gamma actin associated with the contractile phenotype. The authors proposed that the switch from a proliferative to a syntethic phenotype may be regulated by caspase 3 and that the swich from the syntethic to the contractile phenotype by a decrease in progesterone.[74] A concept of emerging importance is that mechanical factors such as uterine stretch can modify the uterine[74,75] and membrane phenotype[76] and play an important role in parturition.

Cervical Ripening

The ability of the cervix to retain the conceptus during pregnancy is unlikely to depend on a traditional muscular sphincteric mechanism.[77,78] The normal function of the cervix during pregnancy depends on the regulation of the extracellular matrix. The major macromolecular components of the extracellular matrix are collagen, proteoaminoglycans, elastin and various glycoproteins such as fibronectins. Collagen is the most important component of the extracellular matrix and determines the tensile strength of fibrous connective tissue.[79] Changes in cervical characteristics during pregnancy have been attributed to modifications in collagen content and metabolism. Proteoaminoglycans have also been implicated in cervical physiology. The proteoaminogylcan decorin (PG-S2) has a high affinity for collagen and can cover the surface of the collagen fibrils, stabilizing them and promoting the formation of thicker bundles of collagen fibers. In contrast, PG-S1 (biglycan) has no affinity for collagen and can therefore disorganize collagen fibrils. The predominant dermatan sulphate proteoglycan in the nonpregnant state is PG-S2, and PG-S1 in the term-pregnant state.[80]

The biochemical events that have been implicated in cervical ripening are: (1) a decrease in total collagen content; (2) an increase in collagen solubility (probably indicating degradation or newly synthesised weaker collagen); and (3) an increase in collagenolytic activity (both collagenase and leukocyte elastase).

Contrary to what is generally believed, extracellular matrix turnover in the cervix is high, and, thus, the mechanical properties of the cervix can change rapidly. Uldbjerg et al[79] demonstrated the importance of collagen content in cervical dilatation, reporting a strong correlation between the collagen content (measured by hydroxyproline determination) of cervical biopsies obtained after delivery and the time required for the cervix to dilate from 2 to 10 cm.

The changes in extracellular matrix components during cervical ripening have been compared to an inflammatory response.[81] Indeed, during cervical ripening there is an influx of inflammatory cells (macrophages, neutrophils, mast cells, eosinophils, etc.) into the cervical stroma. It has been proposed that these cells produce cytokines (e.g., interleukin-8 [IL-8]) and other mediators (such as prostaglandins), both of which have an effect on extracellular matrix metabolism.[82-86]

The chain of events leading to the physiological cervical ripening has not been defined. However, strong evidence supports a role for sex steroid hormones and includes: (1) intravenous administration of 17β-estradiol induces cervical ripening; [87] (2) estrogen stimulates collagen degradation in vitro;[88] (3) progesterone blocks estrogen-induced collagenolysis in vitro;[88] (4) administration of progesterone receptor antagonist induces cervical ripening in the first trimester of pregnancy;[89] and (5) IL-8 production by the uterine cervix is downregulated by progesterone.[90] A detailed review of the mechanisms responsible for the changes in cervical physiology during parturition has been recently published by Norman et al.[91] The reader is referred to this excellent review for further details.

Prostaglandins have been widely used to ripen the cervix prior to induction of labor or abortion. Within hours of administration, prostaglandin E_2 (PGE_2) produces clinical and histological cervical changes resembling physiological ripening that normally develop over several

weeks of gestation. The mechanism of action of PGE_2 is thought to involve stimulation of collagenolytic activity and synthesis of PG-S1 by cervical tissue.[80]

Another mediator implicated in the mechanisms of cervical ripening is nitric oxide (NO),[92] which accumulates at sites of inflammation and can act as an inflammatory mediator at high concentration.[93-95] Expression of the inducible form of nitric oxide synthase (iNOS) and NO production in the cervix increases during labor in animals.[96] Moreover, the administration of L-nitro-arginine methylester (a nonspecific inhibitor of iNOS) blocks cervical ripening,[96] whereas direct application of sodium nitroprusside, an NO donor, to the cervix can induce cervical ripening.[97] Similar results have been obtained in human clinical trials.[98-101]

Cervical changes precede the onset of labor, are gradual, and develop over several weeks.[102] Wood et al[103] were the first to report that a short cervix was a risk factor for preterm labor and delivery. This observation has been subsequently confirmed by several investigators using both manual and sonographic examination.[104-114]

Although cervical shortening is traditionally considered an irreversible change, anecdotal clinical observations indicate that some women can have a short cervix, remain pregnant for a long time and even deliver at term. Reversibility of cervical ripening has been demonstrated with pharmacological intervention in the pregnant ewe. Dexamethasone administration induces cervical ripening and increased uterine contractility. These effects can be reversed with large doses of progesterone.[115]

Decidual/Fetal Membrane Activation

The term 'decidual/membrane activation' refers to a complex set of anatomical and biochemical events resulting in the separation of the lower pole of the membranes from the decidua of the lower uterine segment and, eventually, in spontaneous rupture of membranes and delivery of the placenta. Untimely activation of this mechanism leads to preterm prelabor rupture of membranes (PROM), which accounts for 40% of all preterm deliveries.

Histological studies of the membranes in women with term PROM indicate that membranes ruptured prematurely have a decrease in the number of collagen fibers, disruption of the normal wavy patterns of these fibers, and deposit amorphous materials among them.[116] Similar changes have been observed in the membranes apposed to the cervix in women undergoing elective cesarean section at term with intact membranes.[117] The implication is that although spontaneous rupture of membranes normally occurs at the end of the first stage of labor, the process responsible for this phenomenon begins before the onset of labor.

Studies of the site of membrane rupture have demonstrated a 'zone of altered morphology' (ZAM).[117,118] A significant decrease in the amount of collagen types I, III or V and an increased expression of tenascin have been reported in the ZAM. Tenascin is characteristically expressed during tissue remodeling and wound healing. Its identification in the membranes thus intimates the presence of injury and a wound healing-like response. Observations by Bell and Mallak[119,120] suggest that the changes in the ZAM are more extensive in the setting of preterm PROM. These morphological and biochemical observations are consistent with the outcomes of biophysical studies suggesting that rupture of membranes results from the application of acute or chronic stress on localized areas of the membranes that are weaker.

Structural extracellular matrix proteins, such as collagens, have been implicated in the tensile strength of the membranes, while the viscoelastic properties were attributed to elastin.[121,122] Dissolution of extracellular cements (i.e., fibronectins) is thought to be responsible for the process that allows the membranes to separate from the decidua after the birth of the infant. Indeed, oncofetal fibronectin can be found in the vagina during both term and preterm parturition.[123-126] Therefore, degradation of the extracellular matrix, assessed by the detection of oncofetal fibronectin, is part of the common pathway of parturition.

The precise mechanism of membrane/decidual activation remains to be elucidated. However, roles for matrix-degrading enzymes and apoptosis have been proposed. Several studies have demonstrated increased availability of matrix metalloproteinase-1 (MMP-1) (interstitial collagenase),[127] MMP-8 (neutrophil collagenase),[128] MMP-9 (gelatinase-B) and neutrophil

elastase[129] in the amniotic fluid of women with preterm PROM compared with that of women in preterm labor with intact membranes. Similar findings have been demonstrated in studies of the chorioamniotic membranes after PROM.[130] Plasmin has also been implicated in this process,[126] as this enzyme can degrade type III collagen, fibronectin and laminin.[131] Other MMPs are likely to be involved, but systematic studies have not been conducted to date.[132-134] A role for tissue inhibitors of MMPs (TIMPs) has also been postulated.[135]

Accumulating evidence suggests that apoptosis is important in the mechanisms of membrane rupture.[136,137] Rupture of membranes has been associated with overexpression of pro-apoptotic genes and decreased expression of anti-apoptotic genes.[138] Some MMPs, such as MMP-9, may induce apoptosis in amnion.[139-141] Bell and McParland have recently reviewed the subject in detail.[142]

The Role of Prostaglandins

Prostaglandins have been considered the key mediators for the onset of labor[143-158] since they can induce myometrial contractility,[145-148] changes in extracellular matrix metabolism associated with cervical ripening,[143,144,149-151] and decidual/membrane activation.[159]

The evidence traditionally invoked to support a role for prostaglandins in the initiation of human labor includes: (1) administration of prostaglandins can induce early or late termination of pregnancy (abortion or labor);[152,160-170] (2) treatment with indomethacin or aspirin can delay spontaneous onset of parturition in animals;[171-174] (3) concentrations of prostaglandins in plasma and amniotic fluid increase during labor;[14,175-184] (4) intra-amniotic injection of arachidonic acid, the precursor for prostaglandins, induces abortion;[154] (5) prostaglandin concentrations in amniotic fluid increase early during spontaneous labor at term[183] and in women with PROM at term,[184] and have also been reported in nonhuman primates;[14] and (6) amniotic fluid prostaglandin concentrations increase before the onset of spontaneous labor in humans.[185] Therefore, most of the available evidence in humans, as well as in animals, suggests that prostaglandin concentrations increase prior to the onset of labor or during early labor.[14,183-190] Moreover, in all species examined thus far, including marsupials, prostaglandin secretion increases during labor.[8,191]

Prostaglandins are produced by intrauterine tissues including amnion,[192-194] chorion,[193] decidua,[195,196] myometrium,[197-200] and placenta.[190,193] Although there are many stimulants of prostaglandin biosynthesis, the precise signals responsible for their increase during the course of labor have not been determined. Candidates include cytokines (IL-1, tumor necrosis factor-α),[192,196,201] growth factors (i.e., epidermal growth factor),[202] cortisol,[203,204] and others.

Prostaglandin synthase (PGHS), also known as cyclooxygenase, is the enzyme responsible for the initial step in the production of prostaglandins. PGHS has two isozymes: PGHS1 and PGHS2. Recent reports indicate that there is a topographic variation in the distribution of PGHS2 during labor. Indeed, the mRNA expression of PGHS2 is higher in the lower uterine segment than in the fundal part of the uterus in pregnant baboons. In contrast, the mRNA expression of 15-OH prostaglandin dehydrogenase (PGDH), the enzyme responsible for the prostaglandins' degradation, is reduced in the lower uterine segment compared to that in the fundus. It is possible that these topographic differences in the distribution of these enzymes may play a role in the physiologic control of parturition (see below).[205]

Prostanoid receptors are classified based on the responses to various agonists and antagonists (IP, TP, DP, EP and FP).[191] The EP receptors are classified into four distinct subtypes, two of which stimulate contract responses (EP_1 and EP_3) and two that induce a relaxation response (EP_2 and EP_4).[206] Recent studies have examined the expression of prostaglandin receptors in nonpregnant and pregnant human myometrium, as well as in several animal species.[205,207] Topographic differences in the expression of receptors that stimulate uterine contractility and those that inhibit uterine contractility have been found.[208-210] For example, Smith et al reported an increased expression of EP_2 receptor in the lower uterine segment (which induces relaxation) and increased expression of the EP_3 receptor mRNA in the uterine fundus, while

both express FP and EP_4 in similar quantities.[211,212] These observations are important because PGE_2 elicits different contractile responses in myometrium, depending on the source of the myometrium. PGE_2 generally stimulates the fundus but not the lower uterine segment. Collectively, these findings suggest that there may be functional regionalization of the uterus. However, a subsequent study[213] examining in-vitro contractile responses of the upper and lower segment has not produced findings consistent with those by Smith et al.[211,212]

A Role for the Fetus in the Timing of the Onset of Labor

A role for the fetus in the control of the timing of labor was demonstrated by the classic studies of Liggins et al,[214,215] as well as by the cross-breeding experiments described at the beginning of this Chapter (see Normal Duration of Pregnancy). The importance of the fetal brain has been shown, experimentally, because destruction of the paraventricular nucleus of the fetal hypothalamus results in prolongation of pregnancy in sheep.[216,217] The human counterpart to this animal experiment is anencephaly, which is also characterized by a tendency to have a prolonged pregnancy (if women with polyhydramnios are excluded).[218] However, the situation in human anencephaly is considerably more complicated than the one described in animal experiments. The discrepancy can be explained by the wide range of central nervous system and pituitary lesions found in human anencephaly.

Possible Routes for the Fetus to Signal the Onset of Labor

The effector organs of labor are the myometrium, cervix and membranes. The fetus can signal the onset of parturition/labor by using a set of molecules which could be delivered to the effector organs using the following routes: (1) an endocrine pathway whereby the fetal circulation delivers the molecules to the placenta, and reaches the uterus directly or through the maternal circulation; or (2) a paracrine pathway in which molecules produced by fetal organs, such as the lung or kidneys, reach the amniotic cavity to exert an effect on the fetal membranes which transduce the signal to the myometrium and cervix (Fig. 4).[219]

The current paradigm is that once maturity has been reached, the fetal brain, specifically the hypothalamus, increases CRH secretion, which in turn stimulates adrenocorticotrophic hormone (ACTH) and fetal cortisol production by the adrenals.[220-222] The increase in cortisol in sheep and dehydroepiandrosterone sulphate in primates eventually leads to activation of the common pathway of parturition.[223,224] In the sheep, but not in the human, fetal cortisol induces expression of 17-α hydroxylase, which catalyzes the conversion of progesterone to estrogen. Increasing concentrations of estrogen lead to the transcription of contraction-associated genes in the myometrium that favor the onset of labor. The experiments of Liggins et al[214,215] demonstrated that damage to the fetal adrenal glands or pituitary glands would prolong pregnancy. The administration of a CRH-antagonist has a similar effect.[225] Nathanielsz has proposed that in nonhuman primates which lack 17-α hydroxylase in the placenta, ACTH stimulates the production of androgens that are converted into estrogens by the placenta and initiate the cascade of events responsible for parturition.[226]

Challis and Lye have suggested that the apparent differences in the mechanisms of parturition between the sheep and human can be reconciled.[227] They proposed that cortisol in primates has at least three important functions: (1) it stimulates prostaglandin production by intrauterine tissues directly or indirectly by stimulating the production of PGHS-II; (2) it stimulates the production of CRH by the placenta; and (3) it inhibits the enzyme 15-OH prostaglandin dehydrogenase,[228] which catabolizes prostaglandins.[205,229]

CRH can stimulate prostaglandin production and induce the fetal adrenal glands to secrete DHAS, a source of estrogens that drives the increased expression of oxytocin receptors, gap junctions, and prostaglandins.[230-232] It is important to note that while cortisol suppresses CRH production by the pituitary gland, this hormone stimulates CRH production by the placenta.[228] In support of the importance of cortisol in human parturition, we have reported that fetal plasma cortisol concentrations are elevated in the human fetus before the onset of labor in the

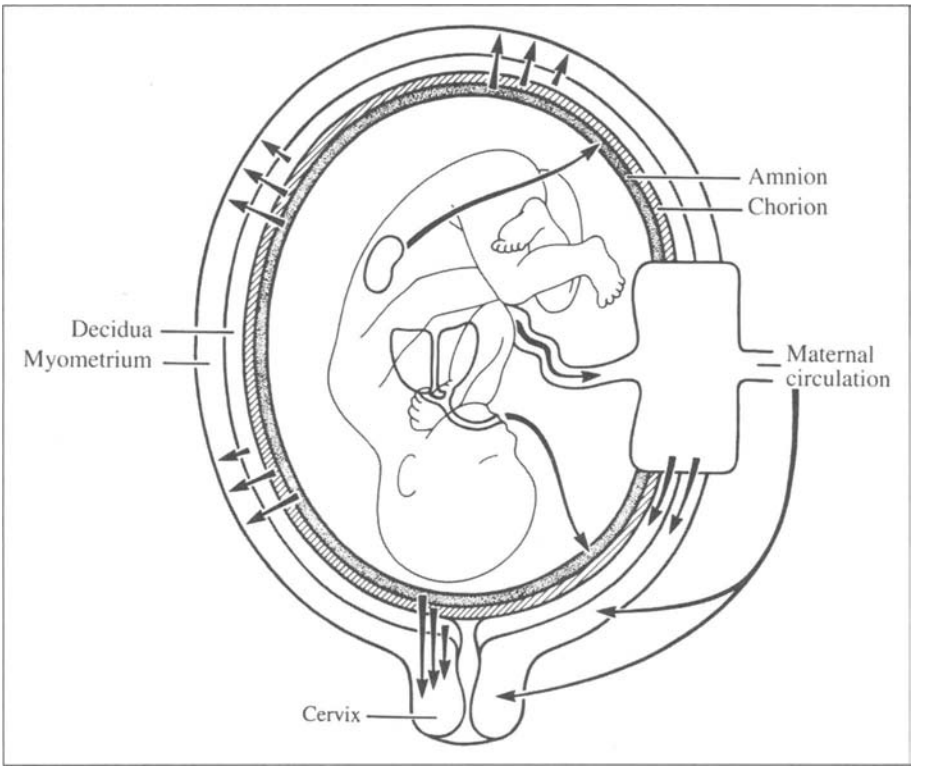

Figure 4. Possible routes of transmission of the signal for the initiation of labor from the conceptus to the mother. Note the large area of contact between the chorion and the decidua which makes the paracrine route an effective way of signal transmission from conceptus to mother. Reprinted with permission from Schellenberg et al.[219]

context of preterm PROM, and that this elevation is associated with the fetal inflammatory response syndrome.[233]

Parturition as an Inflammatory Process

Liggins was the first to compare cervical ripening with an inflammatory response.[214] Since then, accumulating evidence supports that inflammation can be detected in the cervix, myometrium, chorioamniotic membranes and the amniotic cavity in women in labor. Spontaneous labor at term is associated with infiltration of inflammatory cells in these tissues[234-236] and increased production of a wide range of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α, IL-8)[237-239] and chemokines (GRα, MCP-1, ENA, GCSF, GMCSM, neutrophil attractant/activating peptide-1/IL-8, Macrophage inflammatory protein-1α).[239-244] Recently, using a genome-wide screen, Haddad et al were able to demonstrate that genes involved in the control of inflammation are upregulated in the chorioamniotic membranes after labor at term, even in the absence of histologic chorioamnionitis. Of interest is that an inflammatory signature was not observed in the maternal circulation in patients in spontaneous labor at term.[245] This suggests that the inflammatory process is localized to the gestational tissues, and specifically to the membranes. These observations are consistent with the findings of Kim et al, who reported upregulation of the Toll receptor 2 (TLR2) and Toll receptor 4 (TLR4) in normal spontaneous parturition.[246] This observation may reflect that spontaneous parturition is associated with

increased pro-inflammatory cytokine and chemokine response in gestational tissues.[239] Indeed, proinflammatory cytokines have been shown to upregulate the expression of TLR2 and TLR4.[247]

Role of the Placenta

Sixty years ago, Van Wagenen and Newton demonstrated that in pregnant primates, the surgical removal of the fetus, but not of the placenta, does not affect the timing of labor, suggesting that the onset of labor is independent of the presence of the fetus.[248] Similar results were reported by Albrech et al.[249] However, Lanman et al and, more recently, Nathanielsz et al, demonstrated that delivery of the placenta outlasts the normal duration of pregnancy in pregnant rhesus monkeys after fetectomy, indicating that the fetus plays an important role in the timing of delivery in primates, and that in the absence of the fetus, the gestational length is significantly longer than in normal pregnancies.[250,251]

A decade ago, McLean and Smith proposed the existence of a "placental clock," (which is active from an early stage in human pregnancy) and that determines the length of gestation and the timing of parturition.[20] This proposal was based on the result of a large, longitudinal study in which women who delivered preterm had a significantly higher plasma concentration of CRH than those who delivered at term. In contrast, women who had prolonged pregnancies had lower CRH than those that delivered at term. Of note, these differences were already present at 16 to 18 weeks of gestation.[20] The association between a high maternal plasma concentration of CRH and preterm delivery has been confirmed by other studies.[252-254] According to "the placental clock hypothesis," the rate of increase in the maternal plasma CRH through pregnancy determines the timing of labor when saturation of the CRH binding protein is achieved and free CRH becomes available to trigger parturition.[20,255] High concentrations of CRH, described in women with chronic stress, have been associated with a high risk of preterm delivery.[254]

Premature Parturition as a Syndrome

The current taxonomy of disease in obstetrics is largely based on the clinical presentation of the mother, and not the mechanism of disease responsible for the clinical manifestations. The term, "premature labor," for example, does not inform as to whether the condition is caused by an infection, a vascular insult, uterine overdistension, abnormal allogeneic recognition, stress, or some other pathological process. The same applies to other obstetrical complications such as preeclampsia, small for gestational age, fetal death, etc. The lack of recognition of the syndromic nature of obstetric diseases is responsible for the prevailing view that one diagnostic test and treatment will detect and cure, respectively, each of these conditions. The key features of obstetric syndromes[256] are: (1) multiple etiology; (2) chronicity; (3) fetal involvement; (4) clinical manifestations that are often adaptive in nature; and (5) susceptibility owing to gene-environment interaction.

We will review the available evidence to support the concept that premature labor has "multiple etiologies." Clearly, women with a short cervix in the midtrimester of pregnancy or with increased concentrations of fetal fibronectin in vaginal fluid are at increased risk for preterm labor or preterm delivery,[257-260] demonstrating that the pathological disorder leading to these conditions is "chronic" in nature. For example, intrauterine infection can be detected at the time of routine midtrimester amniocentesis for genetic indications and become clinically evident weeks later with either PROM or premature labor.[261-263] "Fetal involvement" has been demonstrated in women with microbial invasion of the amniotic cavity. Fetal bacteremia has been detected in 30% of patients with preterm PROM and a positive amniotic fluid culture for microorganisms.[264] Similarly, neonates born after spontaneous preterm labor or preterm PROM are more likely to be small for gestational age, indicating a preexisting problem with the supply line.[265-271] The "adaptive nature" of labor has been proposed in the context of intrauterine infection in which the onset of labor can be considered a mechanism of host defense against

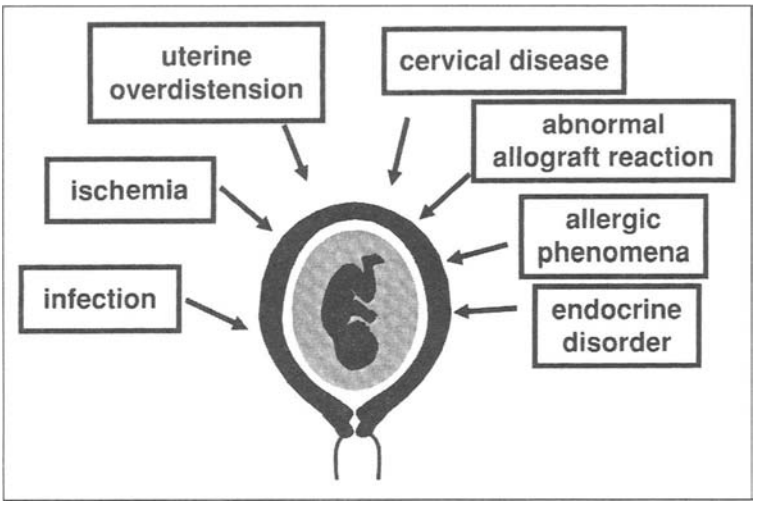

Figure 5. The preterm parturition syndrome. From Romero et al. The preterm parturition syndrome. In: Critchley, Bennett, Thorton, eds. Preterm Birth. RCOG 2004; 28-60.

infection that enables the mother to eliminate an infected tissue and to allow the fetus to exit from a hostile environment.[272,273] It is possible that other mechanisms of disease in premature labor may also threaten the maternal/fetal pair. A key question is why some women develop fetal growth restriction, others develop preeclampsia, and yet another group develops the onset of preterm labor to deal with the problem. If the clinical manifestations are adaptive, then treatment of the components of the pathway (tocolysis, cerclage, etc.) could be considered symptomatic in nature and not aimed at the specific pathological processes that cause preterm labor. Finally, the predisposition to use a mechanism of host defense may be determined by a "gene-environment interaction," as in other disorders such as atherosclerosis and diabetes. However, complexity is added during pregnancy by the presence, and even perhaps the conflicting interests, of two genomes (maternal and fetal).

The pathological processes implicated in the preterm parturition syndrome include intrauterine infection, uterine ischemia, uterine overdistension, abnormal allograft reaction, allergy-induced, cervical insufficiency and endocrine disorders (Fig. 5). The following sections review the evidence for each of the potential mechanisms of disease.

Intrauterine Infection and Inflammation

Intrauterine infection has been recognized as a frequent and important mechanism of disease in preterm birth.[274-277] Indeed, it is the only pathological process for which a firm causal link with prematurity has been established with a defined molecular pathophysiology. Moreover, fetal infection/inflammation has been implicated in the genesis of fetal or neonatal injury leading to cerebral palsy and chronic lung disease.

Evidence of a role of infection in the etiology of preterm birth includes: (1) intrauterine infection or systemic administration of microbial products to pregnant animals can result in preterm labor and delivery;[171,277-286] (2) systemic maternal infections such as pyelonephritis and pneumonia are frequently associated with the onset of premature labor in humans;[287-299] (3) subclinical intrauterine infections are associated with preterm birth;[300] (4) antibiotic treatment of ascending intrauterine infections can prevent prematurity in experimental models of chorioamnionitis;[284,301] and (5) treatment of asymptomatic bacteriuria prevents prematurity.[302]

Microbiological and histopathological studies suggest that infection/inflammation may account for 25 to 40% of cases of preterm delivery.[303] Since infection is frequently difficult to confirm, we often refer to women with histological evidence of acute chorioamnionitis/elevated proinflammatory cytokines in the amniotic fluid and with documented microbial invasion of the amniotic cavity as belonging to "the inflammatory cluster" associated with preterm PROM or preterm labor with intact membranes.

Frequency of Intrauterine Infection in Spontaneous Preterm Birth

The prevalence of positive amniotic fluid cultures for microorganisms in women with preterm labor and intact membranes is approximately 13%.[304] The earlier the gestational age at preterm birth, the more likely that microbial invasion of the amniotic cavity is present.[305] In preterm PROM, the prevalence of positive amniotic fluid cultures for microorganisms is approximately 32.4%.[306] Among women presenting with a dilated cervix in the midtrimester, the prevalence of positive amniotic fluid cultures is 51%.[306] Microbial invasion of the amniotic cavity occurs in 11.9% of twin gestations presenting with preterm labor and delivering a preterm neonate.[307,308] The most common microorganisms found in the amniotic cavity are genital *Mycoplasmas.*

Intrauterine Infection as a Chronic Process

Evidence in support of chronicity of intrauterine inflammation/infection is derived from studies of the microbiological state of the amniotic fluid as well as the concentration of inflammatory mediators at the time of genetic amniocentesis.

Cassell et al[261] were the first to report the recovery of genital *Mycoplasmas* from 6.6% (4/61) of amniotic fluid samples collected by amniocentesis between 16 and 21 weeks of gestation. Two women had positive cultures for *Mycoplasma hominis* and two for *Ureaplasma urealyticum.* Women with *M. hominis* delivered at 34 and 40 weeks without neonatal complications, while those with *U. urealyticum* had premature delivery, neonatal sepsis and neonatal death at 24 and 29 weeks. Subsequently, Gray et al[262] reported a 0.37% prevalence (9/2461) of positive cultures for *U. urealyticum* in amniotic fluid samples obtained during second trimester genetic amniocentesis. After exclusion an elective termination of pregnancy for a fetal anomaly, all women (8/8) with positive amniotic fluid cultures had either a fetal loss within four weeks of amniocentesis (n = 6) or preterm delivery (n = 2). All had histological evidence of chorioamnionitis. These observations suggest that microbial invasion could be clinically silent in the midtrimester of pregnancy and that pregnancy loss/preterm delivery could take weeks to occur. A similar finding was reported by Horowitz et al,[263] who detected *U. urealyticum* in 2.8% (6/214) of amniotic fluid samples obtained between 16 and 20 weeks of gestation. The rate of adverse pregnancy outcome (fetal loss, preterm delivery and low birthweight) was significantly higher in women with a positive amniotic fluid culture than in those with a negative culture (3/6 [50%] versus 15/123 [12%]; $P = 0.035$).

IL-6 concentrations in amniotic fluid are considered to be a marker of intra-amniotic inflammation frequently associated with microbiological infection in the amniotic fluid or the chorioamniotic space.[309-312] Romero et al[313] reported the results of a case-control study in which IL-6 determinations were conducted in stored fluid of women who had a pregnancy loss after a midtrimester amniocentesis and a control group who delivered at term. Women who had a pregnancy loss had a significantly higher median amniotic fluid IL-6 concentration than those with a normal outcome. Similar findings were reported by Wenstrom et al.[314] Of note is that maternal plasma concentrations of IL-6 were not associated with adverse pregnancy outcomes.

The same approach was subsequently used to test the association between markers of inflammation in midtrimester amniotic fluid of asymptomatic women and preterm delivery. The concentrations of MMP-8,[315] IL-6,[316] TNF-α,[317] and angiogenin[318] in amniotic fluid

obtained at the time of midtrimester amniocentesis were significantly higher in women who subsequently delivered preterm than in women who delivered at term.

Collectively, this evidence suggests that a chronic intra-amniotic inflammatory process may be present in early pregnancy and associated with both miscarriage and spontaneous preterm delivery. Some indirect evidence suggests that such inflammatory processes may be detectable using maternal blood.[319]

Fetal Involvement

The most advanced and serious stage of ascending intrauterine infection is fetal infection. The overall mortality rate of neonates with congenital neonatal sepsis ranges between 25 and 90%.[320-324] The wide range of results reflects the effect of gestational age on the likelihood of survival. One study,[320] which focused on infants born before 33 weeks of gestation, found that the mortality rate was 33% for those infected and 17% for noninfected fetuses. Carroll et al[264] have reported that fetal bacteremia detected by cordocentesis is found in 33% of fetuses with positive amniotic fluid cultures and 4% of those with negative amniotic fluid cultures. Therefore, subclinical fetal infection is far more common than traditionally recognized.

Preterm Labor and Preterm PROM as "Adaptive Responses"

We have proposed that the onset of preterm labor in the context of intrauterine infection is a host defense mechanism with survival value. The hosts (mother and/or fetus) would signal the onset of labor for the mother to get rid of infected tissue and maintain reproductive fitness. The molecular mediators involved in parturition are similar to those that have evolved to protect the host against infection (cytokines and other inflammatory mediators).

The current view is that during the course of an ascending intrauterine infection, microorganisms may reach the decidua where they can stimulate a local inflammatory reaction as well as the production of proinflammatory cytokines, chemokines, and inflammatory mediators (platelet activating factor, prostaglandins, leukotrenes, reactive oxygen species, NO, etc.). If this inflammatory process is not sufficient to signal the onset of labor, microorganisms can then cross intact membranes into the amniotic cavity, where they can also stimulate the production of inflammatory mediators by resident macrophages and other host cells within the amniotic cavity. Finally, microorganisms that gain access to the fetus may elicit a systemic inflammatory response syndrome, characterized by increased fetal plasma concentrations of IL-6[273] and other cytokines,[325-327] as well as cellular evidence of neutrophil and monocyte activation.[328]

Thus far, evidence for the participation of IL-1 and TNF-α in the mechanisms of preterm parturition is persuasive, although the precise role of other pro-inflammatory mediators remains to be elucidated. Evidence supporting a role for IL-1 and TNF-α in preterm parturition includes the following: (1) IL-1β[192,329,330] and TNF-α[196,331] stimulate prostaglandin production by amnion, decidua and myometrium; (2) human decidua can produce IL-1β and TNF-α in response to bacterial products;[329,330,332] (3) amniotic fluid IL-1β and TNF-α bioactivity and concentrations are elevated in women with preterm labor and intra-amniotic infection;[201,237,238,333] (4) in women with preterm PROM and intra-amniotic infection, IL-1β concentrations are higher in the presence of labor;[237,238] (5) IL-1β and TNF-α can induce preterm parturition when administered systemically to pregnant animals;[334,335] (6) fetal plasma IL-1β is dramatically elevated in the context of preterm labor with intrauterine infection;[336] and (7) placental tissue obtained from women in labor, particularly those with chorioamnionitis, produces larger amounts of IL-1β than that obtained from women not in labor.[337]

There is considerable redundancy in the cytokine network and, thus, it is not clear that a particular cytokine is required to signal the onset of labor. Results of knockout animal experiments suggest that infection-induced preterm labor and delivery occurs in animals lacking the IL-1 type I receptor.[338] However, a recent observation with a double knockout system indicates

that the IL-1 type I and the TNF-α type I receptors are essential mediators for bacterially induced preterm birth (Hirsch E, personal communication).

Microbial invasion from the amniotic cavity into the fetus can lead to fetal infection and a systemic inflammatory response: "the fetal inflammatory response syndrome" (FIRS). FIRS is a subclinical condition originally described in fetuses of mothers presenting with preterm labor and intact membranes and preterm PROM, and operationally defined as a fetal plasma IL-6 concentration above 11 pg/ml.[273] IL-6 is a major mediator of the host response to infection and tissue damage, and is capable of eliciting biochemical, physiological and immunological changes in the host, including stimulation of the production of C-reactive protein by liver cells, activation of T and natural killer cells, etc. Fetuses with FIRS have a higher rate of neonatal complications, and are frequently born to mothers with subclinical microbial invasion of the amniotic cavity.[273] It is believed that fetal microbial invasion results in a systemic fetal inflammatory response that can progress towards multiple organ dysfunction,[339] septic shock, and death in the absence of timely delivery. Evidence of multisystemic involvement in cases of FIRS includes increased concentrations of fetal plasma MMP-9,[326] neutrophilia, a higher number of circulating nucleated red blood cells, and higher plasma concentrations of G-CSF.[325] The histological hallmark of FIRS is inflammation in the umbilical cord (funisitis) or chorionic vasculitis.[340] Newborns with funisitis are at increased risk for neonatal sepsis[341] as well as long-term handicap, including bronchopulmonary dysplasia[342] and cerebral palsy.[343,344]

An important observation is that fetuses with preterm PROM and FIRS are at risk for the impending onset of preterm labor, regardless of the inflammatory state of the amniotic cavity (see Fig. 5).[272] This suggests that the human fetus plays a major role in the initiation of preterm labor. Nonetheless, systemic fetal inflammation, and even fetal injury, may occur in the absence of labor when the inflammatory process does not involve the chorioamniotic membranes and decidua. Examples of this are hematogenous viral infections of the fetus and/or alloimmunization.

Gene-Environment Interactions

Gene-environment interactions are important in the understanding of many complex disorders such as atherosclerosis, obesity, hypertension, etc. A gene-environment interaction is said to be present when the risk of a disease (occurrence or severity) among individuals exposed (to both genotype and an environmental factor) is greater or lower than that which is predicted from the presence of either the genotype or the environmental exposure alone.[345,346] There is now considerable evidence that the inflammatory response is under genetic control. Therefore, the interaction between the genotype of the host and microorganisms is important in determining the likelihood and course of some infectious diseases. An example of such an interaction has been reported for bacterial vaginosis, an allele for TNF-α and preterm delivery.

Bacterial vaginosis is a risk factor for spontaneous preterm delivery.[260] However, randomized clinical trials with antibiotic administration to prevent preterm birth have yielded contradictory results.[347-359] Recently, Macones et al[360] reported the results of a case-control study in which cases were defined as women who had a spontaneous preterm delivery and controls as women who delivered at term. The environmental exposure was symptomatic bacterial vaginosis. The allele of interest was TNF-α allele 2, given that its carriage had been demonstrated to be associated with spontaneous preterm birth.[361] The key observations were: (1) clinically diagnosed bacterial vaginosis was associated with an increased risk for preterm delivery (OR 3.3; 95% CI 1.8-5.9); (2) women who carried the TNF-α allele 2 were also at increased risk for preterm delivery (OR 2.7; 95% CI 1.7-4.5); (3) and women with both bacterial vaginosis and the TNF-α allele 2 had an odds ratio of 6.1 (95% CI 1.9-21) for spontaneous preterm delivery, suggesting that a gene-environment interaction predisposes to preterm birth. Other gene-environment interactions may determine the susceptibility to intrauterine infection, microbial invasion of the fetus, and the likelihood of perinatal injury.

Uteroplacental Ischemia

Histologic studies of the placentas of patients with preterm labor and preterm PROM indicate that after inflammation, the most common type of lesions are vascular in nature and can involve the maternal and fetal circulations.[362] Maternal lesions include failure of physiological transformation of the spiral arteries, atherosis, and thrombosis, while those observed in the fetal circulation include a decreased number of arterioles in the villi and fetal arterial thrombosis. The proposed mechanism linking vascular lesions and preterm labor/delivery is utero-placental ischemia.

Evidence supporting a role for vascular disorders/uteroplacental ischemia as a mechanism of disease leading to preterm parturition includes the following: (1) experimental uterine ischemia in primates is associated with preterm labor and delivery;[363] (2) vascular lesions in decidual vessels attached to the placenta have been reported by Arias et al[364] in 34% of women with spontaneous preterm labor and intact membranes and in 35% of those with preterm PROM, while in only 12% of control women (term gestation without complications);[364] (3) placental abruption is more frequent in women who deliver preterm with intact membranes or rupture of membranes than in those who deliver at term;[364-369] (4) failure of physiologic transformation in the myometrial segment of the spiral arteries has been found in women with preterm labor and intact membranes and preterm PROM.[370,371] This developmental abnormality had been previously associated with preeclampsia and intrauterine growth restriction; (5) women presenting with preterm labor and intact membranes who have an abnormal uterine artery Doppler velocimetry (an index of increased impedance to flow in the uterine circulation) are more likely to deliver preterm than those with normal Doppler velocimetry;[372-375] and (6) the frequency of small-for-gestational-age infants is increased in women delivering after preterm labor with intact membranes and preterm PROM.[265-271] Vascular lesions leading to compromise of the uterine supply line could account for both intrauterine growth restriction and preterm labor.

The molecular mechanisms responsible for the onset of preterm parturition in cases of ischemia have not been determined. A role for the renin-angiotensin system has been postulated, as the fetal membranes are endowed with a functional renin-angiotensin system[376] and uterine ischemia increases the production of uterine renin.[377,378] Angiotensin II can induce myometrial contractility directly[379] or through the release of prostaglandins.[380]

When uteroplacental ischemia is severe enough to lead to decidual necrosis and hemorrhage, thrombin may activate the common pathway of parturition. Evidence in support of this includes (1) decidua is a rich source of tissue factor, the primary initiator of coagulation and of thrombin activation;[381] (2) intrauterine administration of whole blood to pregnant rats stimulates myometrial contractility,[382] while heparinized blood does not (heparin blocks the generation of thrombin);[382] (3) fresh whole blood stimulates myometrial contractility in vitro and this effect is partially blunted by incubation with hirudin, a thrombin inhibitor;[382] (4) thrombin stimulates myometrial contractility in a dose-dependent manner;[382] (5) thrombin stimulates the production of MMP-1,[383] urokinase type plasminogen activator (uPA), and tissue type plasminogen activator (tPA) by endometrial stromal cells in culture.[384] MMP-1 can digest collagen directly, while uPA and tPA catalyze the transformation of plasminogen into plasmin, which in turn can degrade type III collagen and fibronectin,[385] important components of the extracellular matrix in the chorioamniotic membranes;[386] (6) thrombin/antithrombin (TAT) complexes, a marker of in vivo generation of thrombin, are increased in plasma[387] and amniotic fluid[388] of women with preterm labor and preterm PROM; and (7) an elevation of plasma TAT complex concentration in the second trimester is associated with subsequent preterm PROM.[389]

The recognition that thrombin may play an important role in uterine contractility can explain the clinical observations that retroplacental hematomas in early pregnancy are associated with preterm delivery,[390] and also that vaginal bleeding in the first or second trimester is a risk factor for preterm birth.[391-394]

Although some investigators proposed that fetal hypoxemia is a cause of preterm labor, studies with cordocentesis have indicated that fetal hypoxemia and metabolic acidemia are not more frequent in women with preterm labor and intact membranes who deliver preterm than in those who deliver at term.[395] Similarly, Carroll et al[396] have demonstrated that hypoxemia is not more common in fetuses of women with preterm PROM. Therefore, uterine ischemia should not be equated with fetal hypoxemia, and no evidence currently supports that fetal hypoxemia is a cause of preterm parturition.

Uterine Overdistension

The mechanisms responsible for the increased frequency of preterm birth in multiple gestations and other disorders associated with uterine overdistension are unknown.[397-401]

The importance of stretch in the initiation of labor is supported by an experiment conducted in humans in which the uteri of pregnant women with a live term fetus or a dead fetus were distended with a balloon inflated with physiologic saline (up to 300 cc's).[402] Regular uterine contractions occurred in all patients shortly after distension of the balloon, and all delivered within 21 hours. This observation indicated that stretch can induce labor at term, regardless of whether the fetus is alive or dead. Moreover, amniotic fluid concentrations of PG F2α and its stable metabolite in plasma were increased in stretch-induced labor.[402] Recently it has been reported that, in women with twin pregnancies, the presence of polyhydramnios further increases the rate of preterm delivery.[403]

Central questions are how the uterus senses stretch, as well as how these mechanical forces induce biochemical changes that lead to parturition. Lye et al and other investigators have conducted several studies that explore the biochemical consequences of stretch on myometrium.[404-429]

Increased expression of oxytocin receptor,[417] connexin 43,[65] and the *c-fos* mRNA is consistently demonstrated in the rat myometrium near term.[414-416,418,420] However, using a model in which pregnancy is restricted to one of the two uterine horns (by unilateral tubal ligation), the expression of the same genes was substantially lower in the empty horn, but this could be corrected by the distension of the uterus with a 3-mm tube that stretched the horn.[65,417] In another set of studies, progesterone was found to block stretch-induced gene expression in the myometrium.[73] Mitogen-activated protein kinases have been proposed to mediate stretch-induced *c-fos* mRNA expression in myometrial smooth muscle cells.[414-416,418,420]

In addition to the effects of stretch on myometrium, recent studies indicate that stretch can have effects on the chorioamniotic membranes.[149,430-443] For example, in vitro studies have demonstrated an increase in the production of collagenase, IL-8,[86,436] and PGE_2,[435] as well as the cytokine preB-cell colony-enhancing factor.[439] These observations provide a possible link between the mechanical forces operating in an over-distended uterus and rupture of membranes.

Abnormal Allograft Reaction

The fetoplacental unit has been considered nature's most successful semi-allograft, and abnormalities in the recognition and adaptation to fetal antigens has been proposed as a mechanism of disease in recurrent pregnancy loss, intrauterine growth restriction and preeclampsia.[444-446] Chronic villitis has been considered a lesion indicative of "placental rejection" and such lesions have been found in the placentas of a subset of women who deliver after spontaneous preterm labor.[447] We have observed that some women with preterm labor, in the absence of demonstrable infection, have elevated concentrations of the IL-2 soluble receptor.[362] Such elevations are considered an early sign of rejection in nonpregnant patients with renal transplants.[448] Further studies are required to define the frequency and clinical significance of this pathological process in preterm labor. Recently, allorecognition, in which cytotoxicity and rejection reactions are not inevitable consequences of exposure to foreign antigens,[449] has been proposed as a more accurate means of describing the maternal-fetal relationship, and this has

implications for the concept of allograft rejection as a mechanism of disease in obstetrics. A role for complement and natural killer cells in adverse pregnancy outcome has been suggested,[450-460] but it is unclear if these systems are altered in preterm parturition.

Allergy-Induced Preterm Labor

The concept that an allergic-like mechanism (type I hypersensitivity reaction) may be one of the etiologies of preterm birth was proposed in the early 1990s based upon clinical observations and experimental studies.[461-463] Case reports documented that some women had preterm labor after exposure to an allergen, and that a subgroup of patients with preterm labor have eosinophils as the predominant cell in amniotic fluid. Since the presence of eosinophils in body fluids is generally considered an indicator of an allergic reaction, patients with preterm labor and a predominance of eosinophils in the amniotic fluid were proposed to represent a form of uterine allergy. Subsequent to this observation, a large body of experimental evidence has accumulated, providing biological plausibility to this hypothesis. The key observations have been that mast cells are present in the uterus and their products of degranulation can stimulate uterine contractility. Observations that support a role for a type I hypersensitivity reaction in preterm labor include the following:[464-475] (1) the human fetus is exposed to common allergens, such as house dust mite. The allergen Der p 1 has been detected in both amniotic fluid and fetal blood. Moreover, the concentrations of the allergen are higher in fetal than maternal blood;[468] (2) allergen-specific reactivity has been demonstrated in umbilical cord blood at birth and as early as 23 weeks of gestation, indicating that the fetus can recognize the allergen and mount an immune response;[469] (3) pregnancy is considered a state in which there is a preponderance of a TH-2 cytokine response that favors the differentiation of naive $CD4^+$ T cells to the TH-2 phenotype and the production of IgE, the key immunoglobulin required for a type I hypersensitivity reaction; (4) the gravid uterus is a rich source of mast cells, the effector cells of allergic responses;[474] (5) several products of mast cell degranulation, such as histamine and prostaglandins, can induce myometrial contractility;[472,473] (6) pharmacological degranulation of mast cells with a compound called '48/80' induces myometrial contractility;[465,467] (7) incubation of myometrial strips from sensitized and nonsensitized animals with an anti-IgE antibody increases myometrial contractility;[467] (8) human myometrial strips obtained from women known to be allergic to ragweed demonstrate increased myometrial contractility when challenged in vitro by the allergen (Garfield RE, personal communication). Moreover, sensitivity of the myometrial strips of nonallergic women can be transferred passively by preincubation of the strips with human serum (Garfield RE, personal communication); and (9) premature labor and delivery can be induced by exposure to an allergen in sensitized animals and can be prevented by treatment with a histamine H1 receptor antagonist.[466]

In summary, there is clinical and experimental evidence that a type I hypersensitivity reaction can induce preterm parturition, although the frequency of this phenomenon in humans is still unknown.

Cervical Insufficiency

Although cervical insufficiency (formerly known as cervical incompetence) is traditionally considered a cause of midtrimester miscarriage, accumulating evidence suggests it can also be a cause of spontaneous preterm birth.[476] Cervical insufficiency may be the result of a congenital disorder (e.g., hypoplastic cervix or diethylstilboestrol exposure in utero), surgical trauma (e.g., conization resulting in substantial loss of connective tissue) or traumatic damage to the structural integrity of the cervix (e.g., repeated cervical dilatation associated with termination of pregnancy).[477] Some cases presenting with the clinical characteristics of cervical insufficiency may be due to infection. Indeed, intrauterine infection has been demonstrated in 50% of women presenting with acute cervical insufficiency.[306]

Endocrine Disorders

Steroid hormones, such as progesterone, estrogen, androgens, glucocorticoids, and mineralocorticoids, are synthesized and secreted by endocrine cells into the circulation, enter target cells by diffusion and bind to specific intracellular receptors to activate gene expression.[478] Progesterone and estrogens are steroid hormones that play a central role in pregnancy.[479] Estrogens have been implicated in the activation of labor by inducing expression of connexin 43,[69] oxytocin receptors[480] and by formation of gap junctions.[481] Progesterone maintains uterine tranquility[482] by repressing the expression of a range of "pro-labor genes"[72] and inhibits cervical ripening.[483-485] The importance of progesterone in maintaining human pregnancies has been demonstrated by progesterone inhibitors which can induce abortion and parturition.[479,483,486,487] Progesterone binds to intracellular receptors present in human amniotic, decidual, chorionic and myometrial cells.[49,488] The human progesterone receptors (PR) A (769-amino acid; 94 kDa) and B (933-amino acid; 116 kDa) belong to the superfamily of nuclear receptors that coordinate morphogenesis and homeostasis in response to the binding of their ligand.[489,490] Both PRs are transcribed from one gene, in which the two coding potentials are directed by two promoters designated as A and B. Transcription of the PRs is stimulated by estrogens. After receiving the extracellular signals, the transcriptional action of PRs is achieved through activation of functional elements (AF) that allow for contact with the general transcriptional apparatus, which then permit activation of the specific enhancer elements of target genes.[478]

Steroid receptors have several conserved functional domains designated A to F. The N-terminal A/B domain is highly variable in sequence and usually contains a transactivation functional segment, which activates target genes using the core of the transcriptional apparatus or coactivators that mediate signaling to downstream proteins. The D domain may influence DNA binding properties and anchor some corepressor proteins. Domain E contains the hormone-binding domain (HBD) and is involved in dimerization, ligand binding, nuclear localization and interaction with transcriptional coactivators and corepressors. The DNA binding domain (DBD) is contained within the highly conserved region C. Region C is a zinc-finger domain that can fold and allows for a limited number of amino acids to specifically bind to a cognate DNA sequence called progesterone response element (PRE). Domain C is also involved in receptor dimerization.[491]

The human progesterone-progesterone receptor biological system is, therefore, in part regulated by one gene that encodes the expression of two different products (PR-A and PR-B) with specific and antagonistic activities. PR-B homodimers activate transcription, PR-A homodimers weakly activate transcription, and PR-B/PR-A heterodimers inhibit B receptor activity as well as that of other members of the steroid receptor family.[492] As mentioned above, both PRs contain AFs that regulate expression of genes in which a PRE exists. The PR-B transcript has three AF elements (AF-1,2,3), while PR-A is missing the AF-3 element because PR-A is a truncated form of PR-B. The missing part in the PR-A is an upstream sequence near the N-terminal region of the PR-B.[493] PR-B is a stronger transactivator than PR-A due to transcriptional synergism between AF3 and one of the two downstream AFs. In addition, within the N terminus of the PRs and upstream from AF1, there is an inhibitory factor (IF) capable of repressing the activity of AF1 and AF2, but not the distal AF3. Therefore, this IF cannot repress the function of the PR-B receptor but can repress the function of PR-A.[492]

Although the cell-specific mechanistic effect of PR-B and PR-A on the cofactors of the general transcriptional machinery of progesterone-sensitive genes is not known, inhibition of progesterone action is thought to be important for activation of labor. In some mammalian species, a decreased concentration of progesterone in maternal circulation is known to occur prior to spontaneous labor.[494,495] In humans, however, a progesterone withdrawal in maternal circulation has not been demonstrated prior to the onset of labor and several investigators have proposed alternative mechanisms to explain a lack of progesterone action including: (1) binding of progesterone to a high affinity protein that reduces its bio-availability;[496,497]

(2) increased cortisol concentration that competes with progesterone in the regulation of the corticotrophin-releasing hormone;[498] and (3) the conversion of progesterone to an inactive form within the target cell before interacting with the receptor.[499,500] None of these hypotheses have been proven,[501] and current research has focused on the complex modulation of estrogen/progesterone receptor expression and action.[502-504] Indeed, Pieber et al have proposed that induction of PR-A expression at term may have a role in creating a state of functional withdrawal of progesterone by the dominant repressing effect on hPR-B nuclear transcription,[501] and that the activation of nuclear factor kappa beta (NFκB) in amnion represses progesterone action.[488,505-508] Similarly, Allport et al[505] demonstrated constitutive activity of NFκB in human amnion cells at the time of labor, and showed that activation of NFκB induced COX-2 mRNA expression. The investigators concluded that human labor is associated with increased constitutive NFκB activity within the amnion, the function of which is to increase the expression of COX-2, while contributing to a "functional progesterone withdrawal".[505]

Based on these findings, it is tempting to speculate that by prevention of the progesterone action through the modulation of its receptor leads to up-regulation of a range of pro-labor genes. The pathologic mechanism responsible for suspension of progesterone action in preterm parturition remains to be elucidated. In the case of infection, IL-1 and TNF-α can induce activation of NFκB and this, in turn, may affect progesterone function.[505] Future studies could be directed to the delineation of the three-dimensional structure and interaction of the N-terminal regions of the PR-B and PR-A isoforms, as this may provide insights into new therapeutic opportunities.

Estrogens have also been implicated in the activation of labor.[509] Estrogens are mainly synthesized in the ovary, testis, peripheral tissue and placenta during pregnancy through aromatization of androgens.[510] The human estrogen receptors (ER) have also been characterized and are members of the nuclear receptor superfamily. ERs are ligand-activated transcription factors and exist as two major subtypes named ER-alpha (ERα) and ER-beta (ERβ). These two ERs, which are 95% homologous at the DNA binding domain, are transcribed by separate genes (ERβ has been mapped at 14q and ERα at 6q), and each has a different ligand binding affinity and tissue distributions.[511]

ERs also have several functional domains. There is a highly conserved DNA- binding domain C containing two zinc fingers each involved in receptor dimerization and specific DNA binding, and a less well conserved ligand-binding domain E involved in dimerization, ligand binding, nuclear localization and interaction with transcriptional coactivators and corepressors.[489] ER activation is thought to transform the myometrium from a synthetic to a contractile phenotype[74] by modulating the expression of a set of genes that include COX-2 and Oxytocin receptor (OTr).[512] COX-2 encodes the inducible form of cyclooxygenase responsible for prostaglandin production. OTr expression augments myometrial responsiveness to oxytocin.[513] However, during the entire pregnancy the myometrium is exposed to very high levels of estrogens and myometrial activation does not occur due to the repressive action of progesterone in pro-labor genes.

ERα expression is inhibited by progesterone.[514] Mesiano et al reported that ERα is increased in human myometrium after the onset of labor. ERβ expression, on the other hand, was very low and not affected by labor. The authors reported that the increase in ERα was temporarily related to the increased expression in PR-A and the homeobox gene HOXA10.[515] PR-A blocks progesterone action, while HPXA10 is normally inhibited by progesterone.[515] This positive association supports the concept that progesterone responsiveness of the myometrium is decreased in laboring tissue in vivo despite high circulating progesterone concentration and that the contractile myometrial phenotype is regulated by estrogen responsiveness.

Three mouse strains have been created to determine the functions of estrogens and the two ERs. Estradiol action was removed by inactivating the enzyme aromatase (*cyp19*) (ArKO), which converts testosterone to estradiol.[516] The two estrogen receptors were functionally deleted through targeted disruption of the respective genes (ERKO and BERKO). The ArKO

phenotype demonstrated underdevelopment of the genital tract and mammary glands as well as defects in ovulation. Although it was initially assumed that ERα was indispensable for maintenance of life, development of the female ERKO mouse strain[517] has proven that this is not the case. The most likely explanation is the action of ERβ receptor. Both male and female ERKO mice have abnormal gonads and reproductive behavior, and are infertile. Female BERKO mice have reduced fertility due to blocking of ovulation.[518] Studies of the two mouse strains suggest that the two receptors may compensate for the functions of each other, at least to some extent. Mice with double knockout of both the ERα and ERβ genes will be helpful in clarifying if other ER receptors exist in mammalian species.[491] Furthermore, the effect of receptor interplay in cells expressing both ERs has not been evaluated.

The interactions between PR and ER, where the estrogen action is modulated by the inhibitory effect of progesterone in ER, while PR expression is enhanced by estrogen facilitating its depressing effects on gestational tissues, is a biological system where we have gained considerable insight. It is possible that pharmacological interventions to be developed based on this evidence, may ensure an optimal hormonal environment and response from the time of implantation until delivery.

Randomized Clinical Trials of Progesterone and Progestins in Preventing Preterm Delivery

Several randomized clinical trials have been conducted to determine the efficacy of progestational agents in preventing preterm delivery or recurrent miscarriage. Three meta-analyses have reported contradictory results.[16,519,520]

Daya[519] conducted a meta-analysis that included three controlled trials of progesterone treatment in women with recurrent miscarriage and concluded that progesterone treatment was effective in prolonging pregnancy until at least 20 weeks of gestation (OR 3.09; 95% CI 1.28-7.42).

Subsequently, Goldstein et al[520] reported the results of a meta-analysis of 15 trials conducted in women with previous miscarriage, stillbirth or present preterm labor. There was no evidence that progestational agents were beneficial.

The third meta-analysis was conducted by Keirse[16] and included seven trials, six of which enrolled women considered to be at high risk for miscarriage or preterm delivery. In contrast to previous meta-analyses, this one focused on 17 alpha-hydroxyprogesterone caproate (17P), which is the most widely studied progestational agent. The results showed that 17P administration was associated with a reduction in the rate of preterm labor (OR 0.43; 95% CI 0.2-0.89), preterm delivery (OR 0.5; 95% CI 0.3-0.85) and neonatal birthweight of less than 2500 grams (OR 0.46; 95% CI 0.27-0.8). However, there was no significant difference in the rate of perinatal mortality, respiratory distress syndrome and hyperbilirubinemia between women who received 17P or placebo. The administration of 17P did not reduce the rate of miscarriage.

After a long hiatus in this area of clinical investigation, two recent randomized clinical trials have triggered renewed interest in this approach.

The first randomized double-blind placebo-controlled study[15] evaluated the effectiveness of prophylactic vaginal progesterone in decreasing the rate of preterm birth. This study included 142 high-risk singleton pregnancies. Women were considered at high risk for preterm delivery if they had at least one previous spontaneous preterm birth, prophylactic cervical cerclage, or a uterine malformation. Patients were randomized to receive either daily progesterone (100 mg) or placebo by vaginal suppository from 24 to 34 weeks of gestation. Patients allocated to progesterone had a lower rate of preterm birth before the 34th and 37th weeks of gestation. The rate of preterm delivery at both less than 37 weeks and at less than 34 weeks was lower in the progesterone group than in the placebo group (for 37 weeks: progesterone 13.8% [10/72] versus placebo 28.5% [20/70]; $P = 0.03$, and for 34 weeks: progesterone 2.8% [2/72] versus placebo 18.6% [13/70]; $P = 0.002$).

The second double-blind placebo-controlled clinical trial was conducted in the USA by Meis et al.[17] Women with a history of spontaneous preterm delivery were enrolled at 16 to 20 weeks of gestation and randomly assigned in a 2:1 ratio to receive either weekly injections of 250 mg of 17P or weekly injections of an inert oil placebo until delivery or to 36 weeks of gestation. Treatment with 17P significantly reduced the rate of preterm delivery at less than 37 weeks, less than 35 weeks, and less than 32 weeks of gestation [for 37 weeks: 17P 36.3% [111/306] versus placebo 54.9% (84/153); RR 0.66; 95% CI 0.54-0.81; for 35 weeks: 17P 20.6% (63/306) versus placebo 30.7% (47/153); RR 0.67; 95% CI 0.48-0.93; and for 32 weeks: 17P 11.4% (35/306) versus placebo 19.6% (30/153); RR 0.58; 95% CI 0.37-0.91)]. Moreover, neonates born from women treated with 17P had significantly lower rates of necrotizing enterocolitis, intraventricular hemorrhage and need for supplemental oxygen.

The American College of Obstetricians and Gynecologists Committee on Obstetric Practice issued an opinion[521] in November 2003 stating that further studies are needed to evaluate the use of progesterone in women with other risk factors for preterm delivery, such as multiple gestations, short cervical length or those with positive results for cervicovaginal fetal fibronectin. Despite the benefits of progesterone in women with a history of preterm delivery, the ideal progesterone regimen (formulation) is unknown. The 17P used in the US trial is not commercially available at this time.[521] A formulation for vaginal administration of progesterone is commercially available in Europe and the USA.

Conclusions

Term and preterm labor share a common terminal pathway characterized by increased uterine contractility, cervical ripening and degradation of extracellular matrix, which predisposes to membrane rupture. There is growing evidence that parturition is generally a matter of weeks rather than a process of hours. The mechanisms responsible for the initiation of physiologic labor at term have not been determined in humans. Recent studies implicate the production of fetal or placental signals that would activate the common terminal pathway such as surfactant protein A[522] and CRH/cortisol.[523] The evidence presented in this chapter provides support for the concept that preterm parturition is a complex disorder that is syndromic in nature. The predominant clinical presentation of this syndrome (i.e., uterine contractility, preterm cervical ripening without significant clinical contractility, or PROM) will vary depending upon the type and timing of the insult. This conceptual framework has implications for the understanding of the mechanisms responsible for the initiation of preterm parturition, as well as the diagnosis, treatment, and prevention of preterm birth. Since preterm labor is a heterogeneous condition, it is unlikely that one diagnostic modality or one therapeutic intervention will prevent all preterm births. We believe that the way forward requires a systematic examination of the taxonomy of preterm labor, which is now possible using genomic, proteomic and metabolomic techniques.

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CHAPTER 23

Interleukin-1 and Implantation

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Introduction

Infertility and pregnancy wastage affect one of every nine couples in Western Europe and in the United States. The molecular events of embryonic attachment to the endometrial epithelium and subsequent invasion and nidation into the stroma have long been of interest, scientifically to reproductive biologists and clinically to couples with infertility or habitual abortion and to the physicians caring for them. In order to achieve a successful pregnancy in the human, two major conditions have to be fulfilled: during the 4-5 days of transport through the fallopian tube, the embryo must undergo a series of complex maturation processes and, in the same time, a receptive endometrium must have developed. Human endometrium undergoes characteristic cyclic changes of proliferation and secretion and, without embryonic implantation, the endometrium is shed and the menstrual bleeding occurs. Uterine endometrium therefore is the anatomic prerequisite for the continuation of our species and its main purpose during the reproductive age is to communicate with, receive, nourish and protect the implanting blastocyst.[1]

Understanding the factors involved in preimplantation embryo development and embryo-maternal interaction which result in the complex maturation of the embryo and eutopic implantation is crucial for reproductive medicine. Attempts to overcome the low success rates of human in vitro fertilization therapy by increasing the number of embryos per transfer often result in multiple-gestation pregnancies. These are not only associated with increased evidence of maternal and neonatal complications, but are also cause for concern on the part of medical economists. The total costs for delivery and neonatal care for triplet-pregnancies were calculated with US$ 109,765 and assisted reproduction techniques (ART) were responsible for 77% of higher order pregnancies.[2] On the other hand, even by increasing the number of embryos per transfer, the pregnancy rate will never be 100%.

Cytokines and Implantation

The preimplantation embryo produces several factors during its development to signal its presence to the maternal organism. The appropriate interaction between the preimplantation embryo and maternal endometrium is at least partly controlled by paracrine cytokines and this subject is extensively covered by several reviews.[1,3-6] Cytokine- and growth factors and their corresponding receptors have, on the mRNA-level, been detected in blastomeres and in preimplantation embryos from different species as well as in the human endometrium throughout the menstrual cycle. Although it is known that both the endometrium and the preimplantation embryo express several of these cytokine/growth factor-receptor pairs during the time of implantation and although there is general agreement that both, endometrial and embryonic factors are involved in successful implantation, there is only limited knowledge about the actual role of these factors. A better understanding of these factors during early embryonic

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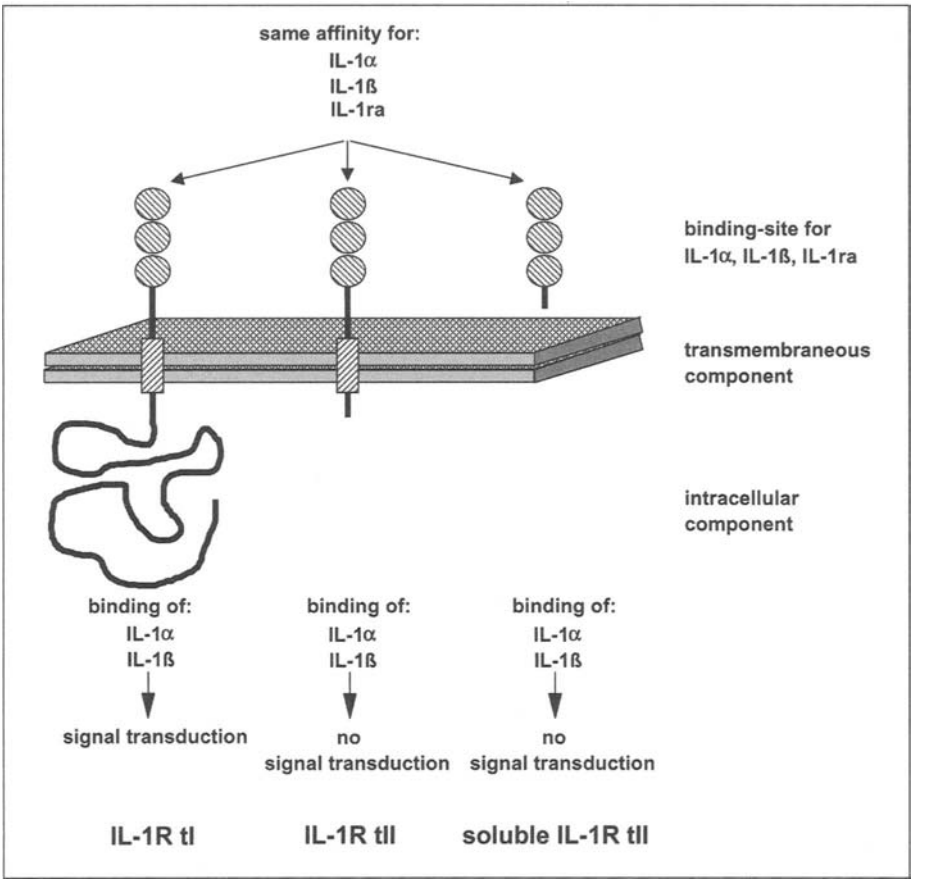

Figure 1. Schematic illustration of the interleukin-1 system. Only binding of either agonist to the IL-1R tI does result in a biological response.[11]

development and implantation could possibly lead to improved in vitro culture conditions and enhance the outcome of human IVF.

The interleukin-1-system (Fig. 1) is composed of two agonists, Interleukin-1α (IL-1α) and interleukin-1β (IL-1β), one antagonist, the Interleukin-1 receptor antagonist (IL-1ra) and two membrane-bound receptors, Interleukin-1 receptor type I (IL-1R tI) and II (IL-1R tII).[7] All components of the IL-1 family in humans are located on chromosome 2 and the protein-, DNA- and RNA-structures are all well characterized for many species. Both agonists are initially synthesized as precursor proteins of 31kDa. The mature proteins have a molecular weight of 17kDa and although the amino acid sequences have a similarity of only ~22%, they induce the same biological responses.[8] There is also a high similarity between the cDNA-sequences of IL-1α and -β in mice and humans.[9] Interleukin-1 receptors type I and II both possess a transmembrane domain and their extracellular portions are homologous with similar binding affinities for the agonists and antagonist; there is also a soluble form of the IL-1R tII. The IL-1 receptor type I is found in low numbers on almost all cell surfaces whereas IL-1R tII is found primarily on white blood cells. Only the binding of either IL-1α or -β to the IL-1 receptor type I results in signal transduction,[10] with receptor type II and the soluble IL-1 receptor acting as competitors of the receptor type I.[11] The IL-1 receptor antagonist binds with a high affinity to both receptors and prevents signal transduction by IL-1α and -β.[12]

The IL-1 system is intimately involved in implantation and preimplantation embryo development. In humans, the IL-1R tI has been detected in total human endometrium[13] and, more specifically, in endometrial epithelial cells with a maximal protein—and mRNA-expression during the luteal phase[14]—the time of embryonic attachment and implantation. IL-1β-mRNA was detected in secretory human endometrium beginning on day 23 of the menstrual cycle.[15] De los Santos et al.[16] detected all major components of the IL-1-system, namely IL-1β, IL-1ra and IL-1R tI immunohistochemically in single human preimplantation embryos.

In a study aimed to develop a suitable methodology for the simultaneous detection of all major components of the IL-1 system in single preimplantation embryos, we could detect all major components of the IL-1 system in single mouse embryos on mRNA-level.[17,18] Also, the frequency of positive mRNA-expression at different developmental stages could be described. The fact that research on human embryos is difficult not only in terms of ethical considerations but also in terms of collecting representative numbers had prompted us to perform our initial investigations on mouse embryos. A total of 231 single preimplantation embryos (11x 2-cell; 48x 8-cell; 58x compacted morulae (CM); 61x early blastocysts (EB); 53x hatching blastocysts (HB)) were examined for the mRNA-expression of a housekeeping gene (β-actin) that served as a positive control and for IL-1β-, IL-1ra- and IL-1R tI-mRNA expression. In 227 (98.3%) of these embryos β-actin was detected. Only embryos with normal morphology and detectable β-actin-mRNA were considered viable and were included in the statistical analysis. Figure 2 representatively illustrates the cDNA-patterns obtained from 12 different preimplantation embryos at various stages of development. In the 2-cell- and the 8-cell-embryos shown here (lanes 2-5), only β-actin could be detected. In one of the two compacted morulae shown here (lanes 6 and 7), an additional cDNA band with a size of 283bp could be detected, representing the mRNA for IL-1ra (lane 6). Two of the three early blastocysts in this figure show a positive IL-1ra-expression (lanes 8 and 9); one shows an additional cDNA band at 844bp, representing a positive IL-1R tI-mRNA-expression (lane 10). Each of the three hatching blastocysts (lanes 11-13) shows a different pattern of mRNA-expression: one HB is positive for IL-1β- and IL-1ra-expression (lane 11), one is positive for IL-1β, IL-1ra and IL1R tI (lane 12) and one is only positive for IL-1ra (lane 13). Lanes 1 and 14 show the 100bp DNA-ladder. The relative numbers of single preimplantation embryos with detectable mRNA-levels for IL-1β, IL-1ra and IL-1R tI at the various developmental stages are also in Figure 2. No mRNA except for β-actin could be detected in 2-cell embryos. Positive IL-1ra expression was first detected at the 8-cell stage (5% of embryos) and the percentage of embryos with a positive IL-1ra-expression subsequently rose from stage to stage (8-cell - CM, CM - HB and EB - HB: $p<0.05$; 8-cell - HB: $p<0.01$ [ANOVA]), reaching a maximum of 74% at hatching blastocyst-stage. IL-1β and IL-1R tI-mRNAs could not be detected in single embryos at the 2-cell- and 8-cell-stage but in a rising percentage of embryos at morula-, early blastocyst- and hatching blastocyst-stage, reaching a maximal percentage at hatching blastocyst stage of 19% for IL-1β and 25% for IL-1R tI, respectively. The increase from 8-cell- to hatching blastocyst-stage was statistically significant ($p<0.05$, ANOVA). In summary, this study demonstrated the presence of IL-1β-, IL-1ra- and IL-1R tI-mRNA in single preimplantation mouse embryos. Increasing embryonic mRNA-expression of cytokines with advancing embryonic development, reaching a maximum in hatching blastocysts could be detected. Coculture of mouse embryos on Vero-cells did not affect these mRNA patterns.[17]

Expression of IL-1 in Human Embryos

In vitro fertilized, cultured human embryos have been shown to produce both IL-1α and IL-1β, and high concentrations (>60 pg/ml and >80 pg/ml) of these cytokines in culture media have been correlated with successful implantation after intrauterine transfer of these embryos,[19] although other authors could not detect IL-1α or -β in culture fluids of human embryos.[20] We have also demonstrated the presence of IL-1β and IL-1R tI -mRNA and -proteins in the

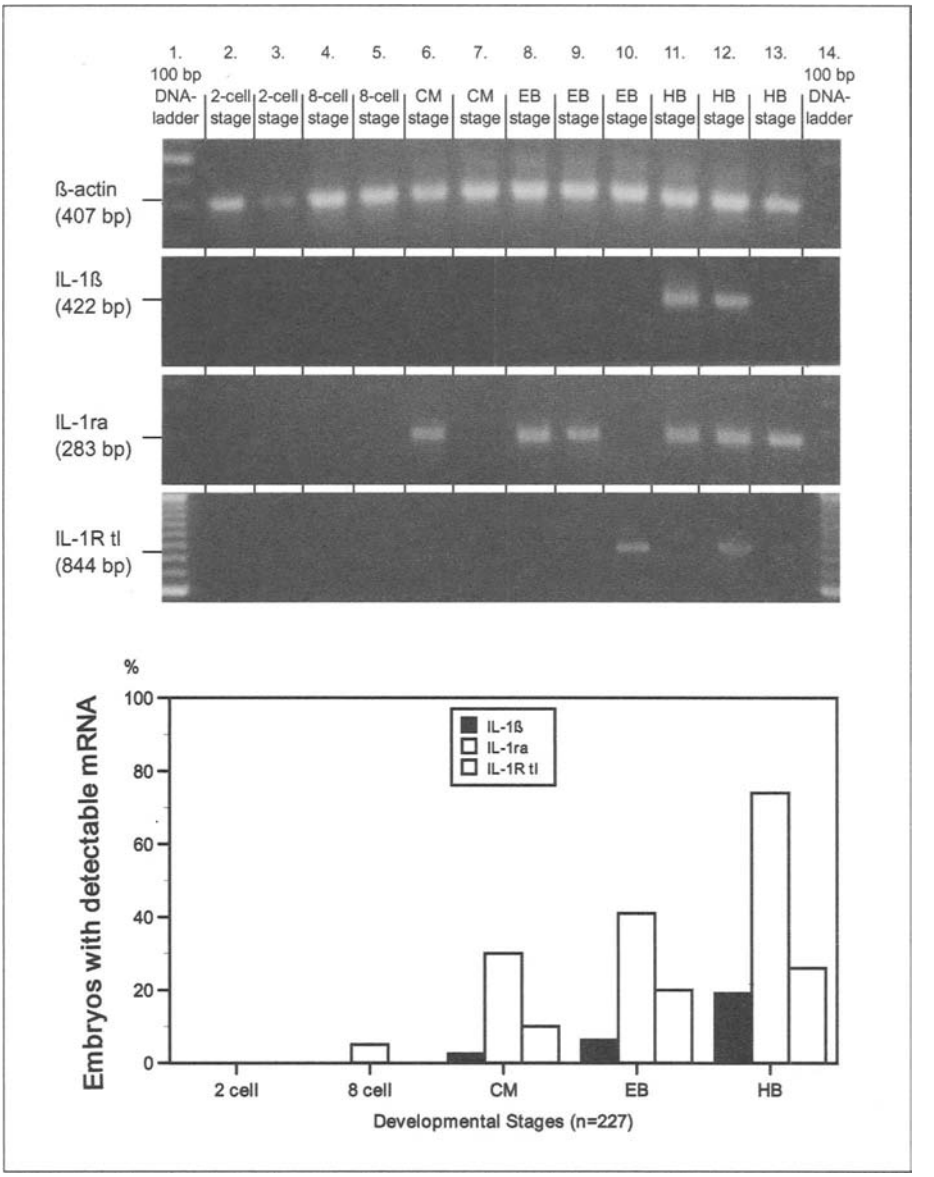

Figure 2. Upper part: Separation of cDNAs after 2 rounds of PCR on 2% agarose-gel stained with ethidiumbromide. Columns 1 and 14: 100bp DNA-ladder. Columns 2-13 represent the results of one single mouse embryo in each column: columns 2 and 3 = 2-cell embryos, columns 4 and 5 = 8-cell embryos, columns 6 and 7 = compacted morulae, columns 8-10 = early blastocysts and columns 11-13 = hatching blastocysts. Lane 1: β-actin-cDNA (407bp), lane 2: IL-1β-cDNA (422bp), lane 3: IL-1ra-cDNA (283bp) and lane 4: IL-1R tI-cDNA (844bp). Lower part: Percentage of mouse-embryos in different stages of preimplantation development with detectable mRNA-expression for IL-1β (black column), IL-1ra (striped column) and IL-1R tI (white column). Modified from Krüssel et al.[18]

human fallopian tube in all phases of the menstrual cycle in epithelial and stromal cells of the human tubal mucosa,[21] as well as the presence of IL-1R tI-mRNA in human preimplantation embryos grown from tripronuclear zygotes.[22] This allows the preimplantation embryo to communicate with maternal surfaces through its IL-1-production during the first 5 days of preimplantation development during tubal transport.

After having shown the expression of IL-1 system mRNAs in preimplantation mouse embryos, we intended to detect the expression of these mRNAs in human preimplantation embryos. We therefore aimed to detect the mRNA-expression of all major components of the IL-1 system in single blastomeres from normally fertilized human preimplantation embryos, and to compare the mRNA pattern with the further development of the biopsied embryo. All patients who chose to participate signed an informed consent approved by the institutional review boards of Stanford University and the Instituto Valenciano de Infertilidad. Human embryos obtained after IVF were cocultured on endometrial epithelial cells as described before[16] until the 8 cell stage, when one embryo from each patient was biopsied using the commonly described procedure to remove one blastomere for the mRNA detection. The single blastomere was examined by one round of RT followed by two rounds of nested PCR using a modification of methods described previously[17,18] for β-actin, IL-1β, IL-1ra and IL-1R tI-mRNAs. Twelve single blastomeres from different patients were examined in this study. All embryos were biopsied at the 8-cell stage and 11 embryos (91.6%) survived the biopsy-procedure as determined by further cleavage after in-vitro culture on human endometrial epithelial cells. Of the surviving 11 embryos, 5 embryos (45.5%) reached the hatching blastocyst stage and were transferred into the recipient's uteri on day 6 after retrieval, together with nonbiopsied embryos from the same patient and the same IVF-attempt. None of these cases resulted in a clinical pregnancy. The mRNA for β-actin that acted as the positive control was detected in all the blastomeres examined. Furthermore, it could be shown that all major components of the IL-1 system were expressed and could be detected in single blastomeres obtained from human embryos cultured in vitro. IL-1R tI-mRNA was detected in all blastomeres (100%). Expression of the IL-1R tI during these early stages of preimplantation embryo development may allow the embryo to receive signals during its passage through the fallopian tube via IL-1β and/or IL-1ra. IL-1β could be detected in 9 of the blastomeres (75%) and IL-1ra was expressed in only 2 (17%) of the blastomeres, those were simultaneously positive for IL-1β. All embryos (100%) that corresponded to the IL-1ra positive blastomeres were arrested in development before reaching blastocyst stage and none (0%) of the blastomeres of embryos developing into hatching blastocysts expressed IL-1ra. IL-1β-mRNA was expressed by 3/5 (60%) of blastocysts from the hatching embryos and in 5/6 (83.3%) of blastocysts from the arrested embryos. Thus far, embryos expressing IL-1ra mRNA in a detectable amount appear to be arrested in early developmental stages. This study demonstrated for the first time that the mRNAs of the major components of the IL-1 system (IL-1β, IL-1ra and IL-1R tI) are expressed and can be detected simultaneously in single blastomeres from normal human preimplantation embryos.

The Role of IL-1 during Implantation

In mice, IL-1α and IL-1β have been detected and localized at mRNA- and protein-level in endometrial endothelial cells[24] in increasing levels from day 3 of pregnancy peaking between days 4 and 5[25] with blastocyst implantation known to occur late on day 4. Systemically administered recombinant human IL-1ra given intraperitoneally from day 3 to day 6 of pregnancy inhibited embryonic implantation in mice,[26] therefore suggesting a role of the agonist (IL-1α and/or β) for attachment. Furthermore it was demonstrated, that this prevention of embryonic implantation by IL-1ra in the mouse seems to be mediated by an effect on the endometrial epithelium, not on the preimplantation embryo. The mechanism by which IL-1ra interferes with embryonic attachment seems to be a direct effect on the endometrial epithelium by inhibition of transformation of the epithelial plasma membrane at the time of implantation, presumably related to the alteration of α4, αv, and β3 adhesion molecules.[27]

The expression of the IL-1-system mRNAs described in these studies may have possible implications for preimplantation embryo physiology and implantation. IL-1β and IL-1ra in human preimplantation embryos may influence the uterine endometrium in a paracrine manner and there are several mechanisms by which this may affect the process of implantation. IL-1 is known to induce the adhesion of some white blood cells like eosinophil and neutrophil granulocytes to endothelial cells[28] and this effect can be specifically antagonized by IL-1ra.[29] One possible mechanism for the attachment of the embryo to the endometrium may be the appropriate regulation of adhesion-molecules such as integrins at the implantation site. There is evidence that integrins, especially $\alpha_v\beta_3$-integrins that are expressed in human endometrium at days 20-24 of the menstrual cycle, may be necessary for embryonic attachment. They have therefore been considered a marker of uterine receptivity.[30] A recent study demonstrated that the β_3-integrin-subunit on the surface of human endometrial epithelial cells (EEC) could be upregulated by the coculture with a human preimplantation embryo. Furthermore, this effect was also achieved when IL-1α and/or IL-1β were added to the EEC-culture and blocked by administration of IL-1β plus anti-IL-1-antibody.[5] It was concluded that the appropriate stimulation of the IL-1R tI by binding of IL-1α and/or IL-1β might be responsible for initiating appropriate endometrial epithelial integrin expression and therefore might trigger the attachment and implantation. After attaching to the uterine surface, the embryo must traverse the epithelial layer and basement membrane to implant in the decidua for further development. The invasion process is associated with tissue remodeling of extracellular matrix and is regulated in part by matrix metalloproteinases (MMPs).[31,32] The 92 kDa collagenase type IV[33-35] and their naturally occurring specific inhibitors, the tissue inhibitors of metalloproteinases (TIMPs)[36] seem to play an important role in these processes. Recently the influence of the interleukin-1 system on this invasion process was demonstrated:[37] the mRNA expression as well as the protein activity as determined by zymogram analysis of the 92 kDa collagenase type IV in cultured human luteal phase endometrial stroma cells was upregulated by coculture with IL-1β in a dose dependent manner. This effect could be reversed by simultaneous coculture with anti-IL1β-antibody and/or IL-1ra. The mRNA-expression of TIMP I and TIMP III in the same cell cultures were downregulated by coculture with IL-1β in a dose dependent manner and this effect could be reversed by simultaneous coculture with anti-IL-1β-antibody and/or IL-1ra. Coculture of luteal phase endometrial stroma cells with transforming growth factor β_1 (TGF-β_1) had a reverse effect on the mRNA expression patterns of the 92 kDa collagenase type IV, TIMP I and TIMP III, therefore acting as a counterbalance to IL-1β and limiting the potential for endometrial invasion. It was concluded from these data that the IL-1β synthesized by the trophoblast cells might modulate the embryonic invasiveness into the maternal endometrium, allowing the embryo to digest the extracellular matrix and the basement membrane.

The IL-1 System as a Regulator of Implantation

The IL-1 system is a major regulator of local cellular interactions during embryonic implantation. Because IL-1β and IL-1ra are both expressed in human endometrium, we hypothesized that an appropriate ratio of IL-1β to IL-1ra might favor the process of embryo implantation. Therefore, we investigated IL-1 regulation of the quantitative ratio of IL-1β/IL-1ra messenger RNA (mRNA) expression in human endometrial stromal cells using quantitative competitive PCR, as well as intracellular protein expression after stromal cell solubilization. Confluent stromal cell cultures were stimulated with human IL-1β (0-1000 IU/mL) for 24 h. After 24 h, total RNA was extracted, reverse transcribed, and coamplified by PCR with a defined amount of internal standard. The quantitative ratio was determined by the density of target to the internal standard. After culture with IL-1β for 24 and 48 h, stromal cells were solubilized, and the intracellular protein levels of IL-1β and IL-1ra were measured by enzyme-linked immunosorbent assay. The IL-1β and IL-1ra mRNA were both up-regulated, and IL-1R tI mRNA was down-regulated, by IL-1β in a dose-dependent manner. The quantitative ratio of IL-1β to IL-1ra mRNA was constant with the presence of increasing concentrations of IL-1β (1-1000 IU/mL).

IL-1β and IL-1ra protein was not detected in conditioned media of cultures before addition of IL-1β. IL-1β and IL-1ra protein levels increased with increasing amounts of IL-1β after solubilization of stromal cells. The IL-1β was detectable after 12 h of culture, in comparison with IL-1ra, which was detectable after 24 h of IL-1β stimulation. The IL-1 agonist and antagonist mRNA were both up-regulated by IL-1β to maintain a constant ratio. Most interesting was the observation that, although intracellular IL-1β protein levels increase linearly with increasing IL-1β concentration (0 -1000 IU/mL), the amount of IL-1ra seems to reach a maximum of approximately 100 pg/mL at 10 IU/mL of IL-1β. Thus, high concentrations of IL-1β may indeed result in an increasing ratio of IL-1β/IL-1ra on a protein level. Although measurements in conditioned media did not show secretion of IL-1β or IL-1ra protein, the mechanism by which these cytokines may modulate implantation could result from release of cytokines by lysis of stromal cells during the implantation process. Additionally, cytokines could be bound to the external surface of the cell membrane, thus allowing them to function as autocrine or paracrine effectors. These results suggest that IL-1 may play a crucial role in embryo-maternal interaction by regulating stromal cell expression of IL-1β and IL-1ra, resulting in an appropriate ratio during the process of embryonic implantation.[38] Other possible mechanisms by which the IL-1 system might influence implantation include a recently investigated interaction with GnRH. IL-1β was shown to specifically up-regulate GnRH-mRNA expression in endometrial stromal cells, allowing a possible role for the mediation of trophoblast invasion and placental hormone regulation.[39]

It should be mentioned that, although there appears to be certain evidence for the important role of the IL-1 system in murine and human reproduction, the IL-1R $tI^{-/-}$ knockout mice, even while having smaller litter sizes when compared to the wildtype IL-1R $tI^{+/+}$ mice, were able to reproduce.[40] Transgenic models are excellent tools to examine functions driven by single genes. This, however, is not the case for most reproductive functions, which are based in redundancy from the processes of implantation to parturition. Implantation is one example wherein redundant mechanisms are critical. Transgenic models therefore cannot be considered the ultimate validation in physiologic processes of reproduction that depend on redundancy for the survival of the species.[27] The absolute necessity for embryonic implantation has only been demonstrated for LIF in the mouse; but clearly, other cytokines and growth factors have important functional roles in this process. Further studies on cytokine and growth factor expression in the endometrium und in preimplantation embryos will provide a better understanding of their role in human infertility and pregnancy lass, and open the way to find new treatment options.

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CHAPTER 24

Immunology and Pregnancy Losses:
HLA, Autoantibodies and Cellular Immunity

Joanne Kwak-Kim, Joon Woo Kim and Alice Gilman-Sachs

Introduction

Pregnancy loss is the one of the most common obstetrical complications. The majority of pregnancy losses are random or isolated incidences that in many cases are related to genetic abnormalities. However, 2-5% of reproductive age women experience recurrent miscarriages.[1,2] Recurrent pregnancy loss is typically defined as two or three or more consecutive pregnancy losses. Genetic, hormonal, metabolic, uterine anatomical, infectious, environmental, occupational and personal habits, thrombophilia, or immune disorders were reported as possible etiologies.[3-6] Despite the many etiologies, a majority of women with recurrent miscarriage have no discernible cause. It has been postulated that immunologic aberrations may be the cause in many of such cases. Immunopathological evaluation of placenta from women with recurrent pregnancy losses of immune etiologies often demonstrate increased inflammatory cell infiltration at the implantation site and increased fibrin deposition on deciduas and/or perivillous placental membrane.[7] In addition, thromboembolism has been noticed in 33.9% of the decidual vessels of the placenta from these women.[7] These findings and others have suggested that inflammation and coagulation play a role in recurrent pregnancy loss.

Pro-inflammatory cytokines play a central role in the differential effects on the coagulation and fibrinolysis pathways.[8,9] Vice-versa, activation of the coagulation system may affect inflammatory responses by direct and indirect mechanisms.[10] In animal models, increased levels of Th1 cytokines activate coagulation by up-regulating the novel prothrombinase fgl2 in a mouse model.[11] In humans, increased proinflammatory cytokines are reportedly associated with changes in the activated protein C (APC) system with a decrease in the ability to generate APC.[12] Pregnant women with increased thrombin reserve and resistance to APC have increased levels of TNF-alpha and this may be important in the risk for adverse pregnancy outcomes.[13] Women with recurrent pregnancy losses and/or implantation failures demonstrate significantly increased peripheral blood T helper 1 cells as compared to normal fertile women.[14] Increased proinflammatory cytokines and up-regulated thrombophilic tendency seems to play a major role in recurrent pregnancy losses. In a mouse model, a physiological cascade of stress is associated with up-regulation of TNF-alpha, and an IL-12-triggered cascade is characterized by persistent up-regulation of TNF-alpha and IFN-gamma as well as a persistent increase in fgl2.[15] In this chapter, possible roles of HLA antigens, autoantibodies, immunocytes and cytokines in inflammatory changes and thrombosis are discussed in relation to recurrent pregnancy losses.

Histocompatibility Gene Products and Their Role in Pregnancy Loss

During a pregnancy the developing fetal placental unit can be considered a semi-allograft, during which allogenic paternal HLA antigens are presented to a mother. Extravillous cytotrophoblasts express nonclassical class I HLA-G and -E molecules,[16] with lowered

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expression of HLA-C antigens even prior to the implantation.[17] One of potential roles of these antigens is the protection of trophoblasts from cytotoxicity. In the mouse bc1- or bc2- (which may be homologous to HLA-G1 and HLA-G2 of human) expressing RMA-S cells were protected from NK cell-mediated rejection in vivo. This finding demonstrates a role for blastocyst MHC in protecting transporter associated with antigen-processing (TAP)-deficient trophoblasts from NK cell attack in vivo.[18] In humans, natural killer cells are inhibited by HLA-G via NKAT3, which contributes to the survival of the fetal semi-allograft in the mother during pregnancy.[19] Despite the experimental data, clinical correlation between HLA-G expression and recurrent pregnancy losses has not been shown.[20] In addition to evidence that HLA-G can inhibit NK cells, there is growing evidence that HLA-G can also regulate T cells. The soluble HLA-G1 isoform downregulates both CD8+[21] and CD4+ T cell reactivity.[22] HLA-G also modulates innate immunity by binding to several NK and/or decidual receptors, inducing secretion of certain cytokines. It is also noteworthy that extraordinary levels of variation in the 5'-upstream regulatory region of HLA-G exist, which may provide evidence for an association between a promoter-region single-nucleotide polymorphism and fetal loss rates. One polymorphism, -725C/G, was associated with fetal loss, with an increased risk for miscarriage in couples in which both partners carried the -725G allele, compared with couples not carrying this allele.[23]

An excess of human leukocyte antigen sharing in primary and secondary recurrent aborters has been reported. The primary aborters shared human HLA- A and DQ antigens and primary and secondary aborters shared three or more of the human leukocyte A, B, DR, and DQ antigens.[24] The role of recessive genes linked to the major histocompatibility complex was speculated on in the pathogenesis of recurrent spontaneous abortions and of gestational trophoblastic tumors. When maternal-fetal histocompatibility for HLA-Class II loci was examined, significantly more couples with RSAs shared 2 HLA DQA1 alleles as compared with fertile control couples. In addition, a significant deficit of HLA DQA1 compatible live born children was observed. It was suggested that HLA-DQA1 compatible fetuses may be aborted early in pregnancy, prior to the time when fetal tissue can be recovered for genetic studies.[25] Prospective studies of pregnancy outcome demonstrate increased fetal loss rates among Hutterite couples matching for HLA-B antigens ($P = 0.033$) or for the entire 16-locus haplotype ($P = 0.002$). HLA region genes have a role in both pregnancy outcome and mate choice, and selective action of these genes may occur preconceptionally as well as during pregnancy.[26]

HLA antigens have been associated with the extent of an immune response to specific antigens. Couples with increased HLA sharing and recurrent pregnancy losses often demonstrate lack of anti-paternal cytotoxic antibodies (APCA), anti-idiotypic antibodies (Ab2) and mixed lymphocyte reaction blocking antibodies (MLR-Bf). Overactivity of T helper-1 (Th-1) cytokines and natural killer (NK) cells have been also reported to be the major alloimmune cause of recurrent spontaneous abortion (RSA).[27] These functions may be associated with specific HLA antigens or genes closely linked to them such as certain complement system proteins or TNFα. HLA class II histoincompatibility has been related with amelioration of rheumatoid arthritis during pregnancy.[28] This finding suggests a histocompatible pregnancy may evoke maternal autoimmunity and perhaps up-regulate T helper 1 immune responses. The underlying pathology of T helper 1 immune responses in histocompatible pregnancies warrants further studies.

Autoimmune Responses

Early in the investigation of autoimmune correlates and recurrent spontaneous abortions, it was noted that some women without systemic lupus erythematosus have positive lupus anticoagulant and histories of fetal losses. These findings led to the question of whether this correlation was the beginning of systemic lupus erythematosus in these women or a new autoimmune entity with gynecoobstetrical manifestations.[29] Autoimmune disorders that affect reproductive processes are often subclinical and most women who present with repeated miscarriage are

otherwise well.[30] Organ-specific antibodies such as anti-thyroid antibodies, and organ-nonspecific antibodies, including antiphospholipid antibody, lupus anticoagulant, anticardiolipin antibodies, antinuclear antibody, anti-ssDNA, anti-dsDNA, and anti-histone antibody have been reported to be associated with pregnancy losses or obstetrical complications.[31-33] Although contradictory results were also reported in regard to the relationship of these antibodies and pregnancy losses, autoantibody associated reproductive failure, characterized by an increased risk of fetal loss and decrease in fecundity appears established. Whether the presence of autoantibodies are direct etiological factors for the pregnancy losses or obstetrical complications, or the epiphenomena, markers of immune activation still need to be explored further.

Antiphospholipid Antibody

The antiphospholipid syndrome (APS) is defined by recurrent pregnancy loss and thrombosis in the presence of antiphospholipid (aPL) antibodies. Experimental data shows that passive transfer of antiphospholipid antibodies result in clinical manifestation of APLS, that is, fetal loss and thrombocytopenia.[34] Antiphospholipid antibodies (aPL) are a heterogeneous group of autoantibodies that are detected by both immunoassays and functional coagulation tests. The antigenic targets are negatively charged phospholipids and serum phospholipid-binding proteins. Despite the strong association between aPL and thrombosis, the pathogenic role of aPL in the development of thrombosis has not been fully elucidated. The most frequently utilized serologic markers for APS are lupus anticoagulant, anticardiolipin antibodies (aCL), and recently anti-beta-2-glycoprotein 1 antibodies.[35] Interestingly, the trophoblast can be targeted by antiphospholipid antibodies (aPL), especially by anti-phosphatidylserine antibody (aPS).[36] Cardiolipin is not present in the trophoblast plasma membrane; nonetheless, anticardiolipin (aCL) has been implicated in trophoblast pathology. Perhaps the crossreactivity between aPS and aCL may have contributed to this pathology.[37]

Previously we have reported that women with recurrent pregnancy losses demonstrated significantly higher prevalence of aCL, aPS and aPE (anti-phosphatidylethanolamine antibody) than those of normal fertile controls.[38] It is noteworthy that the current rheumatological definition of APS does not include aPS or aPE evaluation.[39] In addition, current therapy for pregnant women with APS, which is focused on preventing thrombosis by anticoagulation, is only partially successful in averting miscarriage.[40] Obstetrical features of aPL needs careful investigation from a different perspective based on possible immunopathological effects of aPL on trophoblasts in addition to thrombogenic effect. Proposed mechanisms include antibody-mediated interference with coagulation homeostasis, activation of platelets and endothelial cells and a T-cell immune response to serum phospholipid-binding proteins.[41]

Both animal and in vitro experimental models have shown monoclonal and polyclonal aPS and aCL to specifically destroy trophoblast, inhibit syncytium formation, halt human chorionic gonadotropin (hCG) production, and limit trophoblast invasion. Antibodies to PE (aPE) have not been well characterized: however, recent reports document that aPE are associated significantly with very early (embryonic) recurrent pregnancy loss (RPL). During cytokinesis (late telophase) of Chinese hamster ovary (CHO) cells, formation of PE rafts in cleavage furrows is required for completion of cell division and formation of daughter cells. This raises the question whether aPE might interfere with implantation and cell division during embryogenesis.[42] Recently, elevated plasma adrenomedullin concentration and uterine arterial pulsatility index (PI) were reported in women with antiphospholipid antibodies and recurrent pregnancy losses.[43] Direct effect of the purified IgG positive for anticardiolipin/anti-DNA antibodies from SLE/APS patients with recurrent pregnancy loss (RPL) was reported to reduce rat yolk sac and embryonic growth more than sera negative for these antibodies. These sera reduced cultured human placental trophoblastic cell growth, reduced their proliferation rate and increased their rate of apoptosis.[44]

Complement activation has been reported to be a central mechanism of pregnancy loss in APS. The fact that F(ab)'2 fragments of aPL antibodies do not mediate fetal injury and that C4-deficient mice are protected from fetal injury suggests that activation of the complement cascade is initiated via the classical pathway.[45] When complement activation was blocked, fetal loss was prevented in pregnant mice after injection of antiphospholipid antibodies. Interaction of complement component 5a (C5a) with its receptor is reported to be necessary for thrombosis of placental vasculature.[46]

Recent data demonstrated that intravenous immunoglobulin G (IVIg) inhibits the thrombogenic effects of antiphospholipid antibodies in vivo and reduces the levels of anti-cardiolipin antibodies in the circulation. Blockade of stimulatory Fc gammaR on inflammatory cells is not necessary for this effect. The mechanism of action on IVIg is more likely saturation of the IgG transport receptor, leading to accelerated catabolism of pathogenic antiphospholipid antibodies. These results have implications in the management of thrombosis in antiphospholipid antibodies and have applications for pregnant patients with a history of antiphospholipid antibody syndrome.[47] Each treatment with IVIg resulted in a reduction of anti-cardiolipin antibodies. A partial transient reduction of antiphospholipid antibody levels was observed immediately following each treatment course resulting in an accelerated fetal outcome.[48,49]

Alterations in cellular immunity have been studied in women with RSA who also have positive aPL. CD56+ NK cell levels are significantly elevated in these women as compared to those of women with negative aPL.[50] In addition, lymphocytes from individuals with APS proliferated to β_2GP1 in a serum-free in vitro assay. The response was shown to be Ag-specific, requiring class II molecules, CD4+ T cells, and APCs, and was associated with a selective expansion of CD4+ but not CD8+ T cells. The proliferating T cells produced IFN-gamma but not IL-4, indicating a bias toward a type 1 immune response.[51] These findings indicate a bias toward a T helper 1 immune response in individuals with aPL and may be related to β_2GP1 specificity. aPL binds specifically to apoptotic, but not viable, thymocytes, and that binding is dependent upon the presence of beta 2GPI. Moreover, beta 2GPI binds selectively to the surface of apoptotic thymocytes to generate an epitope for antiphospholipid autoantibodies.[52] Plasma from APS patients up-regulates surface TF expression on normal human monocytes.[53] Moreover, monocytes from patients with primary APS have increased TF Ag and TF-related procoagulant activity that correlates with thrombotic episodes.[54]

Anti-Thyroid Antibody

Six studies have evaluated the relationship between thyroid antibodies and recurrent abortion, defined as three or more spontaneous miscarriages.[33,55-60] The majority of the studies (5/7) reported a statistically significant increase in the incidence of thyroid antibodies in the recurrent abortion group as compared to controls.

The incidence of thyroid antibodies in euthyroid women with recurrent pregnancy loss appears to be significantly increased compared with controls of reproductive age without previous abortions. No correlation between the presence of thyroid autoantibodies and nonorgan specific autoantibodies could be established.[55,56] Organ-specific and nonspecific autoantibodies were speculated to serve independent markers of risk for recurrent pregnancy losses in women with autoimmune abnormalities. The presence of antithyroid antibodies in nonpregnant women with a history of recurrent abortion was reported to identify a subgroup of women at significantly increased risk for yet another pregnancy loss in their next gestation.[61] However, a recent study did not confirm this finding.[62] It has been reported that euthyroid women with thyroid antibodies developed painless thyroiditis within 1 yr of pregnancy loss. This finding suggests that the immunological changes of a short-term gestation may be sufficient to lead to thyroiditis.[63]

Several intervention trials have evaluated the impact of immunosuppressive therapy in women with thyroid antibodies.[64,65] Although all of the trials revealed a decrease in the incidence of recurrent abortion, each study was limited by methodological concerns.[66]

Maternal immune response, trophoblast function, and maternal thyroid function are reported to be correlated. The presence of low concentrations of hCG and free T4 and high levels of TSH and gamma globulins in women with threatened abortion suggests a negative outcome for the pregnancy.[67] However, direct effect of anti-thyroid antibody on trophoblasts has not been investigated. It is speculated anti-thyroid antibody serves as a marker for autoimmune activation and T cell dysfunction. Further study is needed to investigate a possible direct effect of anti-thyroid antibody on trophoblast and its function.

Cellular Immune Responses in Pregnancy Loss

During the development of the fetal-placental unit, the maternal immune system obviously comes in contact with semi-allogenic antigens and systems must be in place to suppress a possible harmful immune response or to enable the growth of the unit without any pathological harm. The immune system that is exposed to these neoantigens consists of lymphocytes in both the peripheral blood (easily sampled) and the endometrium (obviously more difficult to sample). Peripheral blood reflects the systemic immune responses, whereas lymphocytes in the endometrium reflect the local environment. Studies concerning the lymphocyte populations in these two types of compartments have been reported for both normal pregnancies and pregnancy losses.

T Cells in Peripheral Blood

T cells can be characterized by their expression of surface molecules (phenotype) and by their capacity to produce various cytokines. Studies done in women with unexplained pregnancy losses suggest T cell alterations that may be involved in the pathogenesis of recurrent pregnancy loss. In peripheral blood, CD3 positive T cell levels are not different in nonpregnant women with a history of recurrent pregnancy losses as compared to those of normal fertile women.[14,50,68] However, when CD3+ T cell levels are measured during the first trimester of women who miscarried a recent index pregnancy, the levels were significantly lower than those of women who successfully delivered a live infant.[50] Although the exact mechanism has not been studied, it has been suggested that the proportional decrease of CD3+ T cell population in women who miscarried a pregnancy may result from a proportional increase of CD19+ B cells and/or CD56+ NK cells. An increase in either of these cell populations has been associated with pregnancy loss as described below.

It has been suggested that a successful pregnancy is a Th2 cytokine related phenomenon and that Th1 cytokines can be detrimental to a pregnancy.[69] While there is a scarcity of data from human pregnancy indicating that Th1-type immune effectors actually lead to pregnancy loss, there is some compelling evidence linking inappropriate Th1-type immunity to pregnancy loss. The percentages of T helper cell (CD3+/CD4+) levels in women with RSA were not different from those of normal fertile women in our studies.[14,50] Another study demonstrated elevated CD4+ cells in women with RSA.[68] However, CD3+/CD4+ T helper cells can be further characterized by their patterns of cytokine production into T helper 1 and 2 cells. Percentages of peripheral blood Th1 (CD3+/CD4+/TNF-alpha+, CD3+/CD4+/IFN-gamma+) and Th2 cells (CD3+/CD4+/IL-10+, CD3+/CD4+/IL-4+) were determined by flow cytometric analysis in women with histories of recurrent pregnancy losses and normal fertile women. No statistical differences were found between these two groups. However, when the ratios of these cells (Th1/Th2) were determined and compared in these two groups, women with recurrent pregnancy losses had significantly elevated Th1:Th2 ratios compared to that of normal fertile controls.[14] The underlying etiology of a shift in Th1/Th2 polarization needs further investigation.

T suppressor cell (CD3+/CD8+) levels were not different in women with recurrent pregnancy losses compared to controls.[14] The cytokine production pattern of CD3+/CD8+ cells was parallel to that of CD3+/CD4+ cells. The same trend was noticed in CD3+/CD8+ cells in regards to cytokine production. The ratio of CD3+/CD8+/TNF-α to CD3+/CD8+/IL-10 cells

was significantly elevated as compared to those of normal controls. CD8(+)11b(-) cells was significantly higher in patients with recurrent pregnancy loss in comparison with healthy women and T suppressor CD8(+)11b(+) lymphocytes were lower in women with pregnancy failures in comparison with the control group.[68] This compelling study demonstrated significantly elevated Th1 immune responses in peripheral blood lymphocytes of women with recurrent pregnancy losses or multiple implantation failures, which may reflect the systemic contribution of Th1 cytokines. The percentage of CD56+ regulatory T-cells (CD3+/CD56+) in the peripheral blood of patients with a history of recurrent abortion was less than that in the nonpregnant or pregnant women. These results suggest that CD56+ T-cells with extrathymic properties may be associated with the maintenance of normal pregnancy in humans.[70]

In peripheral blood of healthy pregnant women the percentage of T cells with γδ TCR+ was significantly higher ($P < 0.001$) than in that of recurrent aborters or of nonpregnant individuals. These gamma/delta TCR-bearing lymphocytes may have a role in progesterone-dependent immunomodulation.[71] In peripheral blood of healthy pregnant women, the most frequently occurring chain combination was gamma1.4/delta1, whereas in recurrent aborters, the gamma9/delta2 combination was predominant.[72] Thus these two functionally distinct subpopulations are present in the peripheral blood of pregnant women and may also be related to pregnancy outcome.

Endometrial T Cells

The leukocytes that are found in the endometrium or decidua during pregnancy obviously play a role in maintaining a pregnancy. During the menstrual cycle leukocytes progressively infiltrate the endometrium and they may constitute as many as 30% of decidual cells in early pregnancy.[73] CD3+, CD8+, CD4+, TcR αβ+, and TcR γδ+ cells are present in all phases of the menstrual cycle and in early pregnancy in the endometrium. The proportion of these subsets in relationship to total CD3+ T cells is not different between pregnant and nonpregnant human endometrium. However, significantly fewer T cells were detected in endometrium from early pregnancy compared to nonpregnancy tissues.[74] These findings led to the suggestion that endometrial T cells are unlikely to play a significant role in implantation and the maintenance of human pregnancy since they decrease in number considerably in the first trimester of gestation.[74]

A decreased percentage of endometrial CD8+ T lymphocytes and an increased CD4: CD8 ratio has been reported in recurrent aborters. Conversely, recurrent aborters who had normal CD8+ and CD20+ cell numbers and a normal CD4:CD8 ratio subsequently underwent successful pregnancies, while patients with continuing abortions had lymphoid populations similar to those observed in group of habitual aborters group.[75] Elevated $CD4^+$, $CD14^+$, $CD16^+$, $CD56^+$ and MHC class II^+ cells are also reported in women with recurrent miscarriage compared to controls but contradictory to the other report no differences were seen in CD3+ and CD8+ cells.[76] Patients who had miscarriages following endometrial biopsy had significantly more $CD4^+$, $CD8^+$, $CD14^+$, $CD16^+$, and $CD56^+$ leukocytes in their endometrium than either those who had live births or women with proven fertility.[76] It seems that endometrial lymphocytes of recurrent aborters display a distinct immunophenotypic profile and recurrent aborters have altered endometrial immunologic conditions.

Pregnancy is apparently associated with a TH2 environment. CRTH2 is a chemo-attractant receptor for PGD_2 and mediates PGD_2-dependent migration of blood Th2 cells. In a normal pregnancy, $CRTH2^+$ Th2 cells and $CRTH2^+$ Tc2 (cytotoxic) cells are significantly increased at the materno–fetal interface (implantation site) in the decidua. It has been suggested that Th2 and Tc2 cells may be recruited to the materno–fetal interface, at least in part, in a PGD_2-mediated manner.[77] In contrast, in the decidua basalis accumulation of Tc2 cells decreased in recurrent spontaneous aborters with abnormal chromosomes and both Th2 and Tc2 cells decreased in recurrent spontaneous aborters with normal chromosomes. In the decidua parietalis, the

number and percentage of Th2 and Tc2 cells are similar in normal pregnancy, RSA with normal or abnormal chromosome.[78]

Natural Killer Cells

Very early in the study of reproductive immunology, the role of peripheral blood NK cells (CD16+/56+) in the failure of an early pregnancy was suggested. NK cells were shown to recognize trophoblast cells.[79] Although NK cells cannot kill trophoblastic cells in vitro, NK cells activated by cytokines (TNF-alpha, Interferon gamma or IL-2) may kill trophoblast cells in vitro.[80] Indeed a recent study showed that women with recurrent pregnancy losses, infertility and assisted reproductive failures have significantly increased activated peripheral blood NK cell levels (CD56+/69+) compared to normal fertile controls.[81] In addition, activated NK cells can produce cytokines that are abortogenic.[75] Endometrial bed biopsies from women experiencing recurrent pregnancy losses and infertility of unknown etiology reveal an increase in conventional NK cells (CD56+/16+, CD57+ cells).[7,82] Peripheral blood NK cells (CD56+, CD56+/16+) are significantly elevated in women with recurrent pregnancy losses, infertility and assisted reproductive failures as compared with normal control women.[83,84] Quantitation of peripheral blood NK cells in women with recurrent pregnancy losses and infertility of assisted reproductive failures have shown a significant elevation associated with spontaneous abortion of a conceptus of normal karyotype, and abnormal level associated with loss of embryos that are karyotypically abnormal.[50,85,86] Furthermore, increased peripheral blood NK cell cytotoxicity has predictive value for pregnancy losses.[84,87] Thus, conventional NK cells and failure to suppress NK cell activation plays an important role in immunologically preventable spontaneous abortions. Intravenous immunoglobulin G infusion treatment has been shown to downregulate NK cell killing capacity.[83,88] and enhances CD8+ cell activity.[82] Both of these events may be necessary for a successful pregnancy to occur.

Activated NK cells may play a role in implantation failure. Infertile women had a significantly higher expression of the NK cell activation markers CD69+ and CD161+ compared to fertile women.[89] NK cytotoxicity correlated inversely with expression of NK cells bearing the inhibition marker of CD94+. None of the successfully pregnant women of that cycle had elevated levels of NK cytotoxicity whereas 50% of those experiencing a chemical pregnancy loss and those not becoming pregnant had elevated levels of NK cytotoxicity. Thus, immunologic markers can identify mechanisms involved in implantation failure. Activation markers of CD69+ and CD161+ expressed on NK cells as well as NK cytotoxicity can be added to the previously reported risk factors for immunologic implantation failure.[90] A recent report documented an association between increased numbers of circulating NK cells and heavy metal excretion, which may be a causative factor in some cases of subfertility and recurrent miscarriage.[91]

Endometrial NK Cells

Natural killer cells are the most abundant cell population in endometrium and constitute 50-90% of lymphocytes in human uterine decidua in early pregnancy. These cells have a different phenotypic expression (CD16-/CD56^{bright}) than peripheral blood NK cells (CD16+/CD56dim). In term placentas higher percentages of CD56dimCD16+ NK cells and CD56-CD16+ cells were found in decidua basalis whereas the percentage of CD56^{bright}CD16- uterine NK cells was significantly higher in decidua parietalis.[92] These findings suggest functional differences may occur even in different tissues during implantation and growth of the maternal fetal unit.

Recently, a microassay analysis comparing the expression of approximately 10,000 genes in decidual NK cells and peripheral NK cells revealed three-fold changes in 278 of the genes. The greatest number of these genes encoded surface proteins, including the unusual lectin-like receptors, NKG2E and Ly-49L, several killer cell Ig-like receptors, the integrin subunits alpha(D), alpha(X), beta1, and beta5, and multiple tetraspanins (CD9, CD151, CD53, CD63, and

TSPAN-5). Interestingly, two secreted immunomodulatory proteins, galectin-1 and progestagen-associated protein 14, were selectively expressed in decidual NK cells.[93] These findings suggest that decidual NK cells probably have an immunoregulatory role during development of the fetus and genes are activated in that environment during a normal pregnancy.

Infertile women have fewer CD56+ cells than normal fertile controls throughout the luteal phase.[94] In women with recurrent abortions, higher numbers of endometrial CD56+ NK cells were found compared to normal endometrial tissue suggesting a role for CD56+ NK cells in recurrent early pregnancy loss.[95] In a similar study, however, no significant difference in the overall number of endometrial NK cells was reported, although in the same report a higher ratio of $CD56^{bright}$: $CD56^{dim}$ cells was detected in women with recurrent miscarriages as compared to normal controls, suggesting that NK subsets may be important.[75] When 'classic' CD57+ NK cells were investigated, significantly increased numbers of CD57+ NK cells were found in spontaneous abortion cases when compared with normal human pregnancy.[96] CD57+ NK cells at the placental implantation site was significantly increased in 29.6% of women with recurrent pregnancy losses as compared to placental implantation site of elective abortion.[7]

Flow cytometric analysis of decidual lymphocytes from normal pregnancy demonstrated that the relative proportion of decidual NK cells was increased to approximately the same extent in normal, anembryonic pregnancies and recurrent pregnancy losses. Nonetheless, higher decidual NK activity was found in tissue from women with anembryonic pregnancy and recurrent spontaneous abortion than in normal pregnancies.[97] These studies support the notion that increased decidual NK cell activity or numbers is related with pregnancy losses. Interestingly, a decrease of CD56+ cells, was noticed in choriocarcinoma and hydatidiform mole, compared with normal pregnancy, suggesting the necessity of a balance between NK and T cells in controlling trophoblast invasion.[98] Although a direct correlation between the number of CD57+ decidual NK cells and trophoblast invasion has not been studied, 54% of women with recurrent pregnancy losses demonstrated inadequate trophoblast invasion in placental tissue using immunohistochemical techniques that measured depth and the number of cytotrophoblasts.[7]

CD16-$CD56^{bright}$ NK cells that are isolated from total decidual mononuclear cells at the early stage of pregnancy produce many different cytokines such as G-CSF, GM-CSF, M-CSF, TNF-alpha, IFN-gamma, LIF and IL-8. These cytokines may play an important role in a successful pregnancy.[99,100] In addition to these cytokines, HLA-G present on transfected cell lines (mHLA-G) can stimulate vascular endothelial growth factor production by uterine NK cells. These cells also can stimulate proliferation and cytokine production by NK cells, while down-modulating the response of unfractionated uterine mononuclear cells.[101] This finding suggests that the interaction of HLA molecules on trophoblast cells can induce uterine NK cells to produce growth promoting factors or other similar molecules.

The NK cell activity and DNA synthesis of decidual CD16-$CD56^{bright}$ cells was reported to be markedly elevated after IL-2 but not IL-4 treatment.[102] IL-2 stimulation of decidual NK cells was reported to be induced by stem cell factor (SCF). Decidual CD16-$CD56^{bright}$ NK cells express SCF receptor, c-kit but not CD16+ NK cells. Although SCF does not directly influence DNA synthesis in decidual NK cells, it increases the IL-2R alpha expression on CD16-$CD56^{bright}$ NK cells, resulting promotion of NK cell proliferation in response to IL-2.[103] IL-4 inhibits the expression of the IL-2 receptors (IL-2R alpha, IL-2R beta, and IL-2R gamma) on decidual CD16-$CD56^{bright}$ NK cells.[102] This suggests that IL-4 blocks the IL-2-induced NK activity and DNA synthesis of decidual CD16-$CD56^{bright}$ NK cells by inhibiting the expression of IL-2R alpha, beta, and gamma. Thus a TH2 cytokine can affect a TH1 mediated function. TGF-beta 2 hardly affected IL-2-induced NK activation and proliferation.[104]

The importance of regulatory T cells in pregnancy is just being studied in humans. The percentages of NKT cells during pregnancy were significantly increased in the decidua compared with peripheral blood. The percentages of IL-4 and IFN-γ producing NKT cells were significantly higher in the decidua compared with the peripheral blood. These findings suggest that NKT cells might control the Th1/Th2 balance by producing IL-4 and IFN-γ at the feto-maternal interface.[105]

Summary

As new technologies emerge and a better understanding of how the many components of the immune system interact to aid in the growth of the fetus, new treatments will be available to help women with recurrent spontaneous abortions or multiple implantation failures with or without any history of pregnancy loss. Meanwhile, it is clear that local and systemic immunological differences can be found in these women compared to women with normal pregnancies. Some of the data may appear contradictory due to the difficulty in stratifying women with different causes of pregnancy losses. It is important to obtain clinical samples at the optimum time of pregnancy to perform the immunological tests or immunopathological techniques that will benefit these patients. Also, comparisons of the differences in normal pregnancy and pregnancy loss between cytokines, chemokines, homing receptors, hormones, and other molecules that modulate the immune response can only add more information and result in better treatment. However, there is ample evidence that shifts in levels of subsets of peripheral and endometrial NK cells, changes in functional NK cytotoxicity, alterations in cytokine ratios of T helper 1 cells, and the presence of autoimmunity to both organ specific or nonspecific antigens, occur both systematically and locally in recurrent pregnancy losses.

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