Clinical and Developmental Immunology

Immunological Aspects of Human Reproduction

Guest Editors: Raivo Uibo, Andres Salumets, and Gilbert Faure

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Editorial

Immunological Aspects of Human Reproduction

Raivo Uibo,[1,2] Andres Salumets,[2,3] and Gilbert Faure[4]

[1] *Immunology Group, IGMP and Centre of Excellence for Translational Medicine, University of Tartu, 50411 Tartu, Estonia*
[2] *Competence Centre of Reproductive Medicine and Biology, Tiigi 61b, 50410 Tartu, Estonia*
[3] *Department of Obstetrics and Gynecology and IGMP, University of Tartu, 51014 Tartu, Estonia*
[4] *Laboratory of Immunology, Faculty of Medicine, Université Henri Poincaré and Nancytomique, CHU Nancy-Brabois and EA 4369 RHEM, 54500, Vandoeuvre lès Nancy, France*

Correspondence should be addressed to Raivo Uibo, raivo.uibo@ut.ee

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The scientific community of immunologists has been interested in immunological aspects of reproduction since the beginning of last century. Although many questions of reproductive immunology still remain unanswered, several important results have been obtained during recent years. Most importantly, the topic has achieved more wider impact than before. Presently, immunology of human reproduction involves not only the interaction between the maternal immune system and the fetus-placenta status, but also many diverse aspects of reproduction ranging from human gametogenesis to immuno(epi)genetic aspects of diseases of the reproductive system of females and males. Since fertility rates are falling in many countries implying wider usage of various assisted reproduction technologies to overcome reproductive failure, the topic of immunology of human reproduction is receiving increasing attention. Conversely, immunology journals are publishing more papers on every aspect of the immunology of reproduction. The completion of this special issue of Clinical and Developmental Immunology is a good example.

The present issue of this journal is presenting six reviews and eight original papers on diverse immunological aspects of human reproduction.

The first review of S. J. Chen et al. concentrates on abnormalities in the maternal-fetal immunological relationship and the current immunological therapeutic strategies for pathological disorders developing during pregnancy. In the next review paper, K. H. Kikkatalo et al. point out the importance of autoimmune reactions in the development of female infertility and unravel the role of immune reactions against follicle stimulating hormone (FSH) in modulation of female reproductive function. In another review related to hormones and immune reactivity interactions, N. Vrachnis et al. present data on the role of progesterone and corticotropin-releasing hormone (CRH) in myometrium and show their interaction with the immune system during labor. Review paper of M. D. Benson discusses the immunology of amniotic fluid embolism that is one of the leading causes of maternal mortality and morbidity in many countries. In their review about placental IgG transfer in healthy and pathological pregnancies, P. Palmeira et al. analyse the factors participating in IgG transfer. D. V. Vujaklija et al. have chosen to analyse the mechanisms related to cell death at the maternal-fetal interface. In their paper, cytotoxic cells as well as the role of granulysin are under deep scrutiny.

In the original research paper series, the study of J. Calleja-Agius et al. presents the results of the influence of abnormal placental karyotype on inflammatory response evaluated by tumor necrosis factor (TNF) alpha, TNF receptors, and interleukin-10 measurements within villous tissue and blood from women with miscarriage. In the next paper by S. Cardaropoli et al., the fetal growth is studied in connection with macrophage migration inhibitory factor (MIF) and its role in preeclampsia pathogenesis is presented. A. Sarapik et al. bring new data on levels of cytokines, chemokines, and other inflammatory markers in the follicular fluid of patients with different in vitro fertilization (IVF) outcome. The paper of R. Raghupathy et al. presents data about production of pro- and anti-inflammatory cytokines by peripheral blood mononuclear cells stimulated with trophoblast antigens. These

authors show that in case of intrauterine growth restriction, a proinflammatory bias exists in comparison with normal pregnancy. In the paper of M. T. Ahlen et al., the impact of the maternal anti-human platelet antigen 1a (HPA1a or GPIIIa) antibodies in determination of neonatal alloimmune thrombocytopenia is analyzed according to maternal ABO genotypes. W. X. Xu and coauthors are presenting their results on the characterization of B-cell epitopes on human zona pellucida glycoprotein-3, the sperm receptor protein known to have a critical role in fertilization. C. Agostinis et al. have focused their work in assessing the role of mannose binding lectin (MBL) in preeclampsia where this immunologically active substance could also contribute to the endovascular invasion of trophoblast cells. In their paper, W. Zaigui et al. show that functional polymorphism of the gene of Foxp3, a transcription factor involved in regulatory T-cells function, may confer susceptibility to unexplained recurrent spontaneous abortion.

Raivo Uibo
Andres Salumets
Gilbert Faure

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Review Article

Immunologic Regulation in Pregnancy: From Mechanism to Therapeutic Strategy for Immunomodulation

Shyi-Jou Chen,[1,2] Yung-Liang Liu,[3,4] and Huey-Kang Sytwu[2,4,5]

[1] *Department of Pediatrics, Tri-Service General Hospital, National Defense Medical Center, Taipei 114, Taiwan*
[2] *Department of Microbiology and Immunology, National Defense Medical Center, Taipei 114, Taiwan*
[3] *Department of Obstetrics and Gynecology, Tri-Service General Hospital, National Defense Medical Center, Taipei 114, Taiwan*
[4] *Graduate Institute of Medical Sciences, National Defense Medical Center, Taipei 114, Taiwan*
[5] *Graduate Institute of Life Sciences, National Defense Medical Center, Taipei 114, Taiwan*

Correspondence should be addressed to Huey-Kang Sytwu, sytwu@ndmctsgh.edu.tw

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The immunologic interaction between the fetus and the mother is a paradoxical communication that is regulated by fetal antigen presentation and/or by recognition of and reaction to these antigens by the maternal immune system. There have been significant advances in understanding of abnormalities in the maternal-fetal immunologic relationship in the placental bed that can lead to pregnancy disorders. Moreover, immunologic recognition of pregnancy is vital for the maintenance of gestation, and inadequate recognition of fetal antigens may cause abortion. In this paper, we illustrate the complex immunologic aspects of human reproduction in terms of the role of human leukocyte antigen (HLA), immune cells, cytokines and chemokines, and the balance of immunity in pregnancy. In addition, we review the immunologic processes of human reproduction and the current immunologic therapeutic strategies for pathological disorders of pregnancy.

1. Introduction

In 1953, Medawar first proposed the concept of immune tolerance, giving as an example the case of the fetal allograft [1, 2]. He addressed the hypothesis that the semiallogeneic fetus is able to survive because the immunologic interaction between mother and fetus is regulated and inhibited, and that this is because of either a lack of fetal antigen expression resulting from the anatomic separation of the mother from the fetus or a functional suppression of maternal lymphocytes. The exact mechanisms required to induce immunologic tolerance of the fetus are not entirely understood. Despite close contact between fetal trophoblasts and maternal immune cells, there is a lack of antigen stimulation of maternal lymphocytes [3–5].

Successful pregnancy has been considered a biologic example of semiallogeneic graft acceptance, in which the semiallogeneic fetus is protected from immune attack from the mother. Interestingly, the so-called semiallogeneic conceptus actually consists of the trophoblast cells at the maternal-conceptus interface [6]. During the first trimester of human pregnancy, the placenta develops into a dividing villous formation with differentiation of characteristic trophoblast cell types having different functions. Cytotrophoblast progenitors found in the villi follow two differentiation pathways: some fuse to form the multinucleated syncytiotrophoblast layer that encases the floating villi of the placenta, providing the interface with maternal blood to regulate oxygen and protein transport [7], while others follow an invasive pathway and differentiate into extravillous trophoblast (EVT) cells [8]. These cells migrate from the villous tips in columns that anchor the placenta to the maternal decidua, and EVT cells form the cytotrophoblast shield over the decidua as well as migrating and invading the decidua. Invasive EVT cells play an active role in the remodeling processes that occur in the uterine spiral arteries [9]. In human hemochorial placenta, fetal trophoblast cells appear to be in extremely close contact with the maternal immune cells, based on

the observation that EVT cells invade the maternal decidua. Thus, immunologic interrelations between mother and fetus during pregnancy are thought to occur in the decidua [10, 11]. The invasive competence of EVT cells is most dominant during the first trimester and declines afterward.

The major cellular component of the decidua is decidual stromal cells (DSCs). DSCs exert different immune activities that have emerged as relevant to the immunologic interaction between mother and fetus and may lead to either a normal pregnancy or abortion [12, 13]. Recent attention has focused on current knowledge of the effects of pregnancy on the immune response, both peripherally and in the decidua, leading to a discussion on fetal mechanisms for escaping maternal immune attack and the development of immunomodulatory therapeutic strategies for reproductive problems [14]. Understanding the immunologic processes that occur in normal conception will significantly improve our awareness of pathological conditions and suggest strategies to manage associated human reproductive disorders such as abortion, preeclampsia, and preterm delivery [15].

2. Alteration of Human Leukocyte Antigen Expression during Pregnancy

Human leukocyte antigens (HLAs) are also called "transplantation" antigens because they comprise the most powerful stimulators of graft rejection. However, novel HLAs expressed in the fetal membranes are tolerogenic rather than immunogenic [16, 17], and although anti-paternal HLA antibodies are common in pregnant women, they do no damage [18]. One of the fundamental and absorbing paradoxes of life is the immunologic tolerance during reproduction involving the survival and symbiosis of the genetically distinct fetus and its mother [5]. Thus, the mechanisms underlying maternal tolerance are usually effective and raise the essential issue of how immune privilege might be established under the natural conditions of pregnancy to guarantee viability of the embryo/fetus [19–21].

The expression of major histocompatibility complex (MHC) proteins at the interface between mother and fetus is tightly controlled in mammalian pregnancy [22]. The expressed MHC class I genes are subdivided into class Ia, which includes *HLA-A*, *-B*, and *-C* and class Ib, which includes *HLA-E*, *-F*, and *-G*. HLA class II (*HLA-D*) genes are not translated in human trophoblast cells even under inducing conditions where they are transcribed [23, 24] and MHC class II is also undetected in both villous and extravillous human trophoblast cells [25]. Human trophoblast cells express one MHC class Ia molecule (HLA-C) and all three class Ib molecules. In human placenta, the cell-surface expression of MHC class I molecules by the fetal trophoblast is limited to loci of low polymorphism, *HLA-G*, *HLA-E*, and *HLA-C* [26, 27], but the fetal trophoblast cells do not express the MHC class Ia antigens HLA-A and HLA-B [19, 28] that are responsible for the rapid rejection of allografts in humans. The *HLA-C* gene is moderately polymorphic and could possibly stimulate maternal antifetal acquired immunity if the paternal alleles differ from the maternal. Interactions between HLA-C and decidual natural killer (NK) cells may also facilitate trophoblast invasion into maternal tissue: Tilburgs et al. demonstrated that pregnancies with an HLA-C-mismatched child induce an increased percentage of activated T cells in decidual tissue. In addition, HLA-C-mismatched pregnancies exhibit a decidual lymphocyte response to fetal cells and contain functional regulatory T cells in decidual tissue, whereas HLA-C-matched pregnancies do not. This suggests that in uncomplicated pregnancies, decidual T cells exclusively recognize fetal HLA-C at the maternal-fetal boundary, but are prevented from inducing a destructive immune response [29]. Despite this, allelic differences at the HLA-C locus do not seem to be a contributory factor in infertility or termination of pregnancy.

HLA-G was the first of the HLA class Ib molecules expressed by trophoblast cells to be identified and remains an antigen of great interest and a focus of experimental evaluation [19, 20, 30]. Understanding the molecular and biochemical features of the *HLA-G* gene and its products may improve our ability to determine the ways in which HLA-G can affect pregnancy [31]. Multiple reports indicate that levels of HLA-G may predicate reproductive success [20, 32, 33]. As a consequence, fertility physicians are anxious to identify commercially available enzyme-linked immunosorbent assays or other assays that will accurately report levels of HLA-G in the blood of patients with suboptimal fertility [31].

Although it has been proposed that HLA-G may be an evolutionary artifact without function [34], recent studies using HLA-G proteins from transfected cells indicate that these proteins may regulate immune cells and thus may be integral to immune privilege in pregnancy [20, 35]. HLA-G proteins probably target all of the major immune cell subsets [33, 35, 36]. Moreover, Blanco et al. showed that the expression of HLA-G by DSC preserved their potential to control the cytotoxic activity of NK cells against trophoblast and the physiological decay (by apoptosis) of DSC [15].

In the following, we discuss further the complex immune interactions of human reproduction. We describe the activities in pregnancy of lymphocytes, NK cells, uterine (u)NK cells, chemokines, and antigen-presenting cells (APCs) including macrophages.

3. Trophoblast Cells and Immunologic Balance during Pregnancy

An essential issue in pregnancy is how the fetal-placental unit escapes maternal immune rejection. Although fetal and maternal cells interact throughout pregnancy, the fetus naturally continues as a privileged site not subject to rejection [37, 38]. In most of the species that have been studied, expression of MHC molecules by trophoblast cells is repressed, apparently as a strategy to avoid recognition and destruction by the maternal immune system. In recent years, studies of equine pregnancy have advanced the field of reproductive immunology [39]. The trophoblast cells of the horse are unique in the combination of spatial and temporal regulation of MHC expression that they exhibit during placentation. The allantochorion trophoblasts, which comprise the majority of the maternal-fetal interface, do

not express MHC class I proteins, although some mRNA can be detected in these cells [40]. During a short window in early pregnancy, the trophoblasts of the chorionic girdle and endometrial cups transiently express very high levels of polymorphic MHC class I antigens of both maternal and paternal origin [41]. The transcription of both polymorphic and nonpolymorphic MHC class I loci in invasive trophoblasts and the high levels of cell-surface expression of the polymorphic antigens set apart the equine model. Maternal and paternal MHC antigens are both expressed on horse trophoblast, and the mare frequently produces cytotoxic antibodies to the paternal alloantigen shortly after chorionic girdle trophoblast invasion [41]. The expression of HLA-G and HLA-E antigens by the trophoblast may also inhibit cytolysis by natural killer cells [42, 43]. Bacon et al. further demonstrated that, in the equine model, functional alloantigen presentation by the trophoblast can be a normal part of early pregnancy [40].

Peripheral blood lymphocytes isolated from pregnant mares demonstrate a reduced capacity to develop into effector cytotoxic T lymphocytes capable of lysing target cells from the breeding stallion [44]. This reduction in T-cell-mediated alloreactivity reverts after parturition or pregnancy termination and is not observed in males or nonpregnant females. Work by several groups has demonstrated trophoblast-produced soluble factors that may create such an environment by modulating the proliferation and blastogenesis of maternal lymphocytes. Extracts from day 80 placenta have been shown to inhibit the proliferation of maternal lymphocytes, and coculture of chorionic girdle trophoblasts with maternal lymphocytes caused a decrease in proliferation and a reduction in cytokine production [45, 46]. In conclusion, the pregnant mare's immune responses to the trophoblast of her developing placenta are fascinating in their complexity. By providing a window into the nature of maternal-fetal interactions, the horse has illuminated immunologic events not easily detectable in other species.

It is well recognized that there is intimate contact between maternal tissue and the EVT cells that invade the decidua, and that there are high numbers of different types of leukocytes present within the stromal compartment of the luteal-phase endometrium, which increase in first-trimester decidua [10, 47]. Human decidua contain abundant immune cells during gestation, with more than 30% of stromal cells in first-trimester decidua expressing the leukocyte common antigen CD45 [10]. There are four major populations of decidua leucocytes present in early pregnancy: uNK cells, macrophages, dendritic cells (DC), and T cells [48]. Among these, the most abundant are uNK cells ($CD56^+CD16^-$ NK cells), macrophages ($CD38^+CD2^\pm CD3^-CD16^-CD68^+$), and $CD3^+$ T cells ($CD8^+$ and rare $CD4^+$), while B cells are virtually undetectable [11, 49, 50]. The increase in leukocyte numbers during the initial stage of pregnancy suggests that influx and/or proliferation of decidual leukocytes is under endocrine influence. Intimate contact between EVT cells and decidual leukocytes has been demonstrated by electron microscopy and immunohistochemistry, suggesting that there are paracrine interactions between maternal leukocytes and fetal cells [11, 51].

4. NK Cells and uNK Cells in Human Reproduction

In contrast with T and B cells, NK cells do not express somatically rearranged antigen-specific receptors [52]. The functions of NK cells are cell lysis and cytokine production, with individual cells having single or dual competence. For example, lysis is directed against virally infected cells and tumor cells. Interferon (IFN)-γ, which restricts viral infection, is a key cytokine product [53, 54]. The number of peripheral NK cells is decreased in pregnant women compared with nonpregnant women [55, 56], as is IFN-γ production [14].

uNK cells are essentially activated cytokine-producing NK cells [57] that share many characteristics with NK cells. Most peripheral NK cells express the surface marker CD16, an immunoglobulin (Ig) receptor, and have low expression of CD56, an adhesion molecule. In contrast, about 1% of peripheral lymphocytes are $CD16^-CD56^{bright}$ NK cells, and these $CD16^+CD56^+$ NK cells express high amounts of the vascular addressin L-selectin [58]. However, in humans, most uNK cells are $CD56^{bright}$, but lack CD16 and L-selectin [59]. In women, uNK cells differentiate during every menstrual cycle, 3–5 days after the luteinizing hormone surge [60, 61]. The uNK cells may regulate trophoblast invasion into the decidua, myometrium, and uterine spiral arteries [62]. Postmitotic uNK cells are widely distributed within the decidua basalis, commonly (more than one quarter) associated intramurally and intraluminally with spiral arteries. From mid gestation, the number of uNK cells decreases. It appears that during the first half of gestation, uNK cells contribute to and sustain important changes in the maternal placental bed [54] by producing various soluble products including the angiogenic cytokines angiopoietin-2 and vascular endothelial growth factor [62]. In summary, uNK cells are appropriate residents of the maternal-fetal interface because of their unique function in supporting the adaptation of the blood vessels of the pregnant uterus [63].

5. Macrophages in Pregnancy

Macrophages present within the decidual immune cells during pregnancy have the potential to regulate divergent demands: maintenance of immune tolerance toward allogeneic fetal antigens and defense against the constant risk of infection by ascending and blood-borne pathogens [64]. The functional maturation of macrophages has been revisualized in a manner analogous to the well-supported concept of T helper (Th)1/Th2 polarization of effector T cells, by subcategorizing macrophage effector phenotypes as either M1 or M2 [65, 66]. Macrophages activated under the influence of proinflammatory cytokines and lipopolysaccharide are categorized as M1 type, secrete tumor necrosis factor (TNF) and interleukin (IL)-12, and participate in the progression of inflammation. In contrast, M2 macrophages are polarized by exposure to a milieu containing Th2 cytokines (IL-4, IL-10, and IL-13) and glucocorticoids [67]. M2 polarization is characterized by enhanced expression of innate immunity receptors, including scavenger receptors and the macrophage

mannose receptor, as well as an upregulation of arginase activity, which counteracts nitric oxide synthesis [68]. In addition, M2 macrophages exhibit increased secretion of IL-1 receptor antagonist [69] and a reduction in IL-12 production that contributes to the functions of M2 macrophages in tissue repair and anti-inflammation [67]. The M2 polarization of decidual macrophages isolated from normal pregnancies indicates that their immunosuppressive activities are required for the maintenance of immunologic homeostasis during pregnancy. Simultaneously, recognition of hazardous microbes via toll-like receptors (TLRs) and C-type lectin receptors (CLRs) on macrophages is an essential mechanism for host defense in the decidua. Houser et al. propose two distinct subsets of $CD14^+$ decidual macrophages in first-trimester decidual tissue, $CD11c^{HI}$, and $CD11c^{LO}$, which do not fit the conventional M1/M2 categorization [70]. $CD11c^{HI}$ decidual macrophages express genes associated with lipid metabolism and inflammation, whereas $CD11c^{LO}$ decidual macrophages express genes associated with extracellular matrix formation, muscle regulation, and tissue growth. The $CD11c^{HI}$ decidual macrophages also differ from $CD11c^{LO}$ decidual macrophages in their ability to process protein antigens and are likely to be the major APCs in the decidua. Moreover, these populations each secrete both proinflammatory and anti-inflammatory cytokines that may contribute to the balance that establishes maternal-fetal tolerance [70].

The M2 polarization of decidual macrophages isolated from normal pregnancies indicates that their immunosuppressive activities are required for the maintenance of immunologic homeostasis during pregnancy, while the recognition of dangerous microbes via TLRs and CLRs on macrophages is a key mechanism for host defense in the decidua. The remarkable phenotypic plasticity of uterine macrophages allows a balance of these seemingly discrepant activities, and defects in uterine macrophage function are closely linked to the pathophysiology of abnormal gestations, including those complicated by preeclampsia and preterm delivery [64].

6. Immune Tolerance and the Th Milieu during Human Reproduction

A successful pregnancy is the consequence of numerous complex interactions between the receptive uterus and the mature blastocyst under immunohormonal control [71, 72]. The Th1/Th2 ratio reaches a peak in the proliferative endometrium, significantly declines during the secretory phase and is at its lowest level in the early pregnancy decidua [73]. During the early phase of pregnancy, a successful implantation occurs in a proinflammatory microenvironment, and a Th1-type response is followed by a shift to Th2 to control endocrine and immune interactions [74–76]. Several cytokines such as TNF-α and IL-1 induce leukemia inhibitory factor expression in the stroma and epithelial cells, and through their receptors provide paracrine signals to both embryonic tissues and uterine epithelium during implantation [77]. Th1 responses may be suppressed during human pregnancy via downregulation of nuclear factor (NF)-κB and T-bet transcription [78]. In addition, progesterone stimulates a Th2-type response, reduces inflammatory cytokines, and represses (potentially deleterious) allogeneic responses, conferring fetal survival [79, 80].

Decidual $CD4^+CD25^+$ T regulatory cells (Tregs) constitute about 14% of the total decidual $CD4^+$ T cells and express glucocorticoid TNF receptor-related protein, OX40, and cytotoxic T lymphocyte antigen (CTLA)-4 [81]. CTLA-4 expression on Tregs may augment indoleamine 2,3-dioxygenase (IDO) expression by decidual and peripheral blood DCs and monocytes [82]. IDO is involved in maternal tolerance of the fetus by restraining the availability of tryptophan to T cells *in situ* in the uterine microenvironment [83]. Human pregnancy also involves expression of L-arginase, which exhausts arginine in the fetal-placental microenvironment, thus limiting maternal T-cell activity [84].

While uNK cells, macrophages, and DCs aid in orchestrating the balance between pro- and anti-inflammatory milieus over the course of gestation in humans, human decidua has also recently been shown to contain a small population of immature myeloid DCs [85]. Tregs in the uterus are thought to be mainly immunosuppressive. Decidual $CD14^+$ cells express HLA-DR, but low levels of the costimulatory molecules CD80/CD86, suggesting that they could induce Tregs. The current recognized hypothesis predicts that the potential of trophoblastic antigens to induce a natural and tolerogenic maternal response engages Tregs, cytokines, chemokines, IDO, and galectin-1 derived from the fetoplacental unit [86–88], which suggests a possible strategy to treat pathological pregnancy via immunoregulation.

7. The Role of Chemokines in Successful Pregnancy

Chemokines are another important component that are involved in the complex immune network of the fetoplacental unit by adapting normal T cell trafficking and modulating the inflammatory process [89, 90]. We highlight CCL5 (also known as regulated upon activation, normal T cell expressed, and secreted (RANTES)), a proinflammatory chemokine that plays a part in the Th1 response, contributing to a tolerogenic response at immune-privileged sites in murine models, and which might function as an essential modulator of alloantigen-specific T-cell responses during normal pregnancy [91, 92]. Successful pregnancy is accompanied by an increase in RANTES serum levels, whereas these were found to be diminished in patients with recurrent spontaneous abortions [93]. In addition, Ramhorst et al. demonstrated, after treating Ishikawa cells—a human endocervical cell line—with recombinant RANTES and CCR5 (a receptor for RANTES), that there was a decrease in mRNA for CXCR4 (a chemokine receptor associated with a Th2 response) that correlated with an increase in expression of T-bet (the main transcription factor involved in development of a Th1 response) [94]. They also demonstrated that RANTES specifically suppresses alloactivated maternal T cells [95]. Thus, the high levels of progesterone present during normal human pregnancy, particularly at the maternal-fetal interface, would

be predicted to promote RANTES production to levels required for the local induction of a tolerogenic immune response. This would suggest that RANTES may play an important role during maternal-fetal crosstalk, allowing trophoblast cell survival and a maternal tolerogenic response [96].

Evidence obtained either *in vitro* or *in vivo* has shown that three chemokine receptors, structurally related to signaling receptors, but incapable of activating signal transduction, the Duffy Antigen Receptor for Chemokines (DARC), D6, and CCX CKR, act as chemokine decoy receptors [97]. The best-known chemokine decoy receptor is the D6 molecule, a seven-transmembrane domain protein that shares 30%–35% sequence identity with signaling chemokine receptors, but cannot induce known chemokine receptor-signaling functions such as chemotaxis [98, 99]. D6 recognizes the majority of inflammatory CC chemokines and targets them for degradation [99]. D6 is strongly expressed by invading trophoblast cells and on the apical surface of syncytiotrophoblast cells [100]. Interestingly, Wessels et al. demonstrated that D6 is expressed in endometrial epithelium, uterine glands, and trophoblast; furthermore, in a model of spontaneous fetal loss in swine, a marked loss of D6 immunoreactivity was observed in arresting versus viable littermate attachment sites [101]. These results suggest that the absence of the scavenging function of D6 results in increased susceptibility to inflammation-driven fetal loss [102].

8. Immunomodulatory Molecules in Threatened Pregnancy

During pregnancy, the maternal immune system is obviously active and, under certain conditions, may contribute to fetal damage/death. Well-defined pathological processes include destruction of fetal erythrocytes (Rh antigen, erythroblastosis) and platelets (Human platelet alloantigens (HPA)-1 and HPA-2, alloimmune thrombocytopenia) by maternal antibodies and infections during pregnancy, where activated macrophages secreting high levels of Th1-type cytokines alter the fragile cytokine balance at the maternal-fetal interface [20, 103]. Takeshita et al. found that adipsin immunoreactivity was detected either at the decidua basalis in normal placentas or at the placental maze in absorbed placentas [104]. However, they also showed that the quantity of adipsin was increased in the absorbed placentas compared with the normal placentas, suggesting that local expression of adipsin has an effect at the maternal-fetal interface and probably plays a role in spontaneous abortion [104].

uNK cells have been suggested to have a critical function in pregnancy by promoting decidual health, appropriate vascularization of implantation sites, and placental size. In the murine pregnant uterus, extravillous cytotrophoblasts have invaded the maternal decidua. While the decidual macrophages or DCs recognize the trophoblast debris, uNK cells may become active and acquire a cytotoxic function like that of peripheral NK cells, propagating an immune attack on fetal organs and leading to abortion or premature fetal loss [105]. Recently, upregulation of Th17 cells and their related cytokines (e.g., IL-17 and IL-23) was observed in the blood and decidual tissues of patients with unexpected abortion [106]. Wang et al. also demonstrated that the suppressive activity of Tregs on Th17 cells was decreased in patients with unexplained recurrent miscarriages, that the ability of Tregs to repress inflammatory cytokine production may be effected by direct cell-cell contact, and that transforming growth factor-β and IL-10 could inhibit the expression of IL-17 [107]. Thus, it is likely that investigation of immunomodulatory molecules during pregnancy could assist in developing strategies for prevention or treatment of abortion or fetal loss. We herein focus on three immunoregulatory strategies: (1) induction of endogenous peroxisome proliferator-activated receptor-γ (PPARγ) to reduce antioxidant stress and its related immunomodulation; (2) delivery of decoy receptor 3 (DcR3) to neutralize LIGHT (lymphotoxin exhibits inducible expression and competes with herpesvirus glycoprotein D for HVEM on T cells, LIGHT also known as TL4 or TNFSF14)/Fas signaling; (3) overexpression of galectin-9 to block the T-cell immunoglobulin mucin (TIM)-3 pathway and its potential immunomodulatory role in threatened pregnancy.

PPARγ is a member of the nuclear receptor superfamily, a group of transcription factors that regulate expression of their target genes upon ligand binding. Endogenous ligands including oxidized fatty acids and prostanoids can bind to and activate the receptor [108]. Barak et al. reviewed the role of PPARγ in the areas of adipocyte and macrophage biology, insulin action, bioenergetics, and inflammation and somewhat surprisingly found that PPARγ plays an essential role in placental biology [109]. PPARγ may also function in modulating fetal membrane signals toward parturition. In addition, Schaiff demonstrated the unique aspects of PPARγ function in trophoblasts, which may have direct implications for the use of PPARγ ligands during pregnancy [110]: PPARγ agonists may decrease the risk of preterm delivery by suppressing the inflammatory response within the fetal membranes. Additional research that focuses on the mechanism of action, molecular targets, and functions of placental PPARγ is paramount for the translation of these potentially beneficial functions of PPARγ into therapeutic use during pregnancy [110]. Linoleic acid is a well-known component of many foods and is present in vegetables, fruits, nuts, grains, and seeds. Linoleic and linolenic acids are easily absorbed by oral intake to allow bioavailability to the plasma and the brain [111]. The conjugated form of linoleic acid, cis-9, trans-11, and a well-researched PPARγ ligand was shown to be formed naturally from linoleic acid by gut flora, especially probiotics [112]. This suggests that appropriate nutrition, such as linoleic acids and linolenic acids combined with probiotics that are able to upregulate PPARγ ligands, could provide benefits favoring an uncomplicated pregnancy.

TIM glycoproteins share common structural motifs, including a signal peptide, Ig domain, mucin domain, transmembrane domain, and intracellular tail, with phosphorylation sites [113]. TIM-3 was originally identified as a Th1-specific cell-surface molecule that downregulates Th1 responses through inducing apoptosis signaling by galectin-9 engagement [113, 114]. These results suggest that TIM-3 may modulate the Th1/Th2 balance. In addition, recent reports

show that TIM-3 is also expressed on innate immune cells such as DCs and seems to promote innate immunity [115]. Such features of TIM-3 are consistent with the paradigm of Th1/Th2 shift and the activation of the innate immune system in pregnancy. Zhao et al. showed that in pregnant women, TIM-3 enhances both innate and adaptive immune responses by means of its upregulation in innate immune cells, and abnormalities of TIM-3 in pregnant woman may be deleterious to a normal pregnancy. Therefore, TIM-3 may be an indicator for predicting the risk of abortion in pregnant women [116]. In our recent study, we demonstrated that control of the pathogenic Th1 cell immune response through overexpression of galectin-9 to suppress TIM-3 signaling and downregulate proinflammatory cytokine production can inhibit the progressive destruction of β cells in autoimmune diabetes [117], a finding that may suggest a possible strategy for treatment of threatened pregnancy.

A soluble decoy receptor, DcR3, that binds to FasL and inhibits FasL-induced apoptosis has been identified [118], and FasL and LIGHT are established as ligands of DcR3 [119, 120]. Functionally, DcR3 can block FasL/LIGHT-mediated apoptosis leading to the escape of cells from immune attack. TNF-like ligand 1A (TL1A), the third ligand of DcR3, is a costimulator of T cells that promotes IL-2 responsiveness and increases secretion of proinflammatory cytokines both *in vitro* and *in vivo*. In addition, DcR3 suppresses TL1A-induced NF-κB activation and apoptosis [121]. Of note, Gill and Hunt postulate that placental cytotrophoblast cells are protected from LIGHT-mediated apoptosis by both soluble receptor DcR3 and cellular inhibitors of apoptosis-2 to protect human cytotrophoblast cells against LIGHT-mediated apoptosis [122]. In addition, Yen et al. demonstrated that human gestational tissues showed differential production of DcR3, and that decidual DcR3 protein was lower in anembryonic than normal pregnancies [123]. We have shown the immunomodulatory and therapeutic activity of DcR3 in various experimental autoimmune disorders in nonobese diabetic mice [124, 125], experimental autoimmune experimental encephalomyelitis [126], and murine autoimmune crescentic glomerulonephritis [127], suggesting a potential activity of DcR3 in the regulation of successful pregnancies. However, above so-called potential immunomodulatory molecules are only the tips of icebergs in the understandings of the complex immunopathogenic mechanisms of threaten pregnancy. Nevertheless, further studies are essential to clarify these hidden mysteries.

9. Conclusion

The immunologic bond between mother and fetus remains a mystery, although current advances in molecular immunobiology have clarified many of the parameters involved in the fetomaternal interaction during implantation. Experimental models provide major insights in the field of reproductive immunology and the immunomodulation of normal or pathological pregnancy. However, ethical issues concerning the study of the physiology of early pregnancy in humans, together with the difficulty of generalizing animal findings to humans, are basic impediments to the clarification of the implantation process and its subsequent investigation [128].

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Review Article

Review on Autoimmune Reactions in Female Infertility: Antibodies to Follicle Stimulating Hormone

Kadri Haller-Kikkatalo,[1,2,3] Andres Salumets,[2,3] and Raivo Uibo[1,3]

[1] *Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila Street 19, Biomedicum, Tartu 50411, Estonia*
[2] *Department of Obstetrics and Gynecology, University of Tartu, L. Puusepa 8, 51014 Tartu, Estonia*
[3] *Competence Centre on Reproductive Medicine and Biology, Tiigi 61b, Tartu 50410, Estonia*

Correspondence should be addressed to Kadri Haller-Kikkatalo, kadri.haller-kikkatalo@ut.ee

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Female fertility can be affected by diseases or dysfunctions of reproductive tract, neuroendocrine system, and immune system. Reproductive autoimmune failure can be associated with overall activation of immune system or with immune system reactions specifically directed against ovarian antigens. Majority of the antiovarian autoantibodies are directed against β-subunit of follicle stimulating hormone (anti-FSH). This paper summarizes a current clinical classification of female infertility in the context of general activation of autoimmunity and antiovarian autoimmunity by describing serum anti-FSH. The presence of naturally occurring anti-FSH in healthy women will be discussed. In addition, the putative impairment of ovarian folliculogenesis in case of increased production of those antibodies in infertile women will be characterized.

1. Introduction

Infertility is a condition that affects a couple and is defined as the lack of conception after an arbitrary period of 12 months without using any contraception [1]. These couples comprise the infertile and the sterile members of the population, for whom is no possibility of natural pregnancy, and the remainder who are subfertile [2]. The latter inadvertently includes normal fertile females who failed to conceive by chance during the 12 or 13 opportunities a woman has per year [1]. Infertility contributes a great proportion to overall reproductive ill health, since there are ~60–80 million infertile couples (~15% of couples) around the world [2]. Minor fertility impairment is seen in both partners more frequently than expected (>70% of infertile couples) [2]. Although infertility *per se* may not threaten physical health, it may have a serious impact on the mental and social well-being of couples and may result in detrimental social consequences, such as divorce or ostracism [2]. In addition, infertility contributes to low birthrate, which is a major social and national issue in developed countries.

Infertility represents an increasing medical problem. A progressive decrease in fertility rate has been indicated since 1955 [1]. The decrease is associated with both medical and nonmedical factors. Women's age is the major determinant of the average time required to conceive. The highest live birth rates are in the age group of 25–30 years and declines sharply after the age of 35 [3]. Also, the duration of infertility contributes meaningful information to the estimation of future fertility [4]. Chromosomal aberrations, monogenic diseases, endocrine dysfunctions, sexually transmitted diseases (STDs), and immune system dysfunctions are medical situations, which can contribute both to male and female infertility. Unfortunately, still in about 10%–20% of couples, the infertility cause remains unknown [2]. However, autoimmune mechanisms may be the case in those couples and have been associated with premature ovarian failure (POF), "subclinical" ovarian failure and with recurrent pregnancy loss [1].

Nowadays, when the utilization of assisted reproduction technologies has improved the prospects of infertility treatment, still every second infertile couple seeks for

medical advice [2]. First child following *in vitro* fertilization (IVF) was born in 1978 [5]. Today, approximately 2.5% of newborns account for IVF-treated couples in European countries, which remains somewhat lower when compared to Nordic countries [6]. Regardless of constant improvement of pregnancy rate in IVF, the success rates are still around 30% per cycle [6]. Autoimmunity and the presence of autoantibodies have been invoked as a possible mechanism of IVF failure. There are contradicting data regarding the importance of certain antibodies to damage directly the preimplantation embryo, interfering with implantation process or formation of placenta [7–11]. Consequently, the overall activation of the immune system in female infertility has been suggested [12].

For the purpose of improving infertility treatment, the mechanisms of immune system associated with natural reproduction as well as with infertility should be carefully evaluated. This paper summarizes a current clinical classification of female infertility in the context of general activation of autoimmune processes and antiovarian autoimmunity by describing serum antibodies to follicle stimulating hormone (FSH).

2. Autoimmunity

Active tolerance mechanisms are required to prevent inflammatory responses to the many innocuous air-borne and food antigens that are encountered at mucosal surfaces. However, the most important aspect of tolerance is self-tolerance, which prevents the body from mounting an immune attack against its own tissues—prevention from autoimmune reactions. Autoimmunity is associated with a dysbalance of various components of the immune response and with the development of autoantibodies directed against normal host antigens. The susceptibility to autoimmune reactions is regulated at several levels [13]. The proliferation of mature T-lymphocytes in response to either self- or foreign antigenic stimuli is affected by the nature and strength of antigenic peptide-MHC (major histocompatibility complex) stimulation [13, 14]. Human leukocyte antigen (HLA)-class II molecules influence the stability of the antigenic-peptide-HLA complex in an allele-specific manner, affecting the induction of central tolerance [13]. As revealed by the studies on anti-insulin autoimmunity, the stimulation provided by antigenic peptide-MHC stimulation could also be modulated by genetic variations of the insulin gene, influencing the gene expression in the thymus [15, 16]. Tissue-specific autoimmunity appears to be additionally dependent on local factors, including infection-related tissue damage [13], iatrogenic manipulations [17], and the level of autoantigen in periphery [18, 19]. Thus, the expansion of cells responding to low-affinity ligands (self-antigen) or anomalies in the deletion of high-affinity autoreactive T-cells can lead to autoimmune reactions [14]. Once an autoimmune disease has been developed, a wider range of autoimmune reactions may progress, meaning that an individual may develop more than one autoimmune disease [20].

3. Reproductive Autoimmune Failure in Women

Female fertility is regulated by a series of highly coordinated and synchronized interactions in the hypothalamic-pituitary-ovarian axis. Therefore, female fertility can be affected by diseases or dysfunctions of reproductive tract, neuroendocrine system, and immune system or by any severe or exhausting general disease. The etiology of female infertility in a diagnostic and treatment point of view is summarized in Table 1 (based on the guidelines provided by [1, 2]). The reproductive autoimmune failure syndrome was originally described by Gleicher et al. in women with endometriosis, infertility and increased autoantibodies [21]. Autoimmune mechanisms as well as an increased production of multiple autoantibodies are involved in such infertility disorders as POF, endometriosis, polycystic ovary syndrome (PCOS), unexplained infertility, and repeatedly unsuccessful IVF attempts and may be responsible for the pathophysiology of preeclampsia or spontaneous abortions, as stated in many original articles as well as discussed in reviews (Table 2) [19, 22–25]. Although not many studies have been performed on humans, the role of cellular immunity in ovarian autoimmunity, in addition to humoral immunity, has been detected both locally in the ovary [26] as well as in periphery [27]. However, due to the technical difficulties in everyday laboratory work, most clinical studies are restricted to detecting serum antibodies in order to define autoimmune activation in a patient.

In Western Europe and North America, where tubal diseases are relatively uncommon, endocrine dysfunctions can be identified in about 10%–20% of women presenting with infertility [28]. Most common cause for hypergonadotropic hypogonadism is POF [1]. POF is defined as secondary amenorrhea with elevated gonadotrophin levels observed under the age of 40 and affect 1%-2% of women of the general population [1]. POF is highly heterogeneous condition and can be associated with autoimmune disorders, ovarian surgery, iatrogenic causes such as chemoradiotherapy, systemic diseases such as galactosaemia, or with genetic factors [1]. In more than half of the cases, the development of POF has been associated with autoimmune reactions to ovarian tissue [29, 30]. An investigation of antiovarian autoimmune reactions and autoantibodies may be severely hampered by the fact that POF represents an end-stage of disease. By the time when the disease in a women is diagnosed, she has exhausted her follicular supply and, presumably, also the target antigen for the autoimmune attack on her ovary. Thus, the autoimmunity causal of POF can be difficult to detect retrospectively. Regardless of that, high prevalence of antiovarian antibodies (AOA) (30%–67%) and others organ- and nonorgan-specific autoantibodies have been observed in patients with POF [29, 31, 32].

Normogonadotropic anovulation represents about 50% of women with an endocrine cause of infertility and includes mostly the patients with PCOS. PCOS affects up to 4%–10% of all women of reproductive age [33, 34]. PCOS is characterized by polycystic ovaries, oligoanovulation, insulin resistance, and hyperandrogenism or hyperandrogenaemia [35, 36]. Infertility in PCOS is associated with an alteration in

Table 1: Etiology of female infertility (based on the diagnostic and treatment guidelines provided by [1, 2]).

Anovulatory infertility
Hyperprolactinaemia
Pituitary adenoma
Hypogonadotrophic hypogonadism
Kallmann's syndrome
Weight loss
Hypergonadotropic hypogonadism
Premature ovarian failure (POF) and early menopause
Gonadotrophin resistance due to a receptor defect
"Normogonadotropic" oligoanovulation
Polycystic ovary syndrome (PCOS)
Adrenal cause of hyperandrogenism
Genetic determinants
Turner syndrome, Swyer syndrome
Androgen insensitivity syndrome
Androgen synthesis disorders
Tuboperitoneal infertility
Tubal factor infertility
Endometriosis
Autoimmunity
POF
Recurrent pregnancy loss
Autoimmunity-associated infertility
Uterine abnormalities
Malformations
Submucous myomas
Endometrial adhesions
Unexplained infertility

folliculogenesis and in the selection of the dominant follicle leading to anovulation [37]. An autoimmune mechanism has also been suggested in some cases of PCOS, where increased prevalence of AOA and common organ- and nonorgan-specific autoantibodies has been detected [19, 22, 25].

Tubal factor infertility accounts for 10%–30% in developed countries and up to 85% in developing countries of reported cases of infertility [38]. Decreased fecundity may be attributed to impaired ovum transport due to fimbrial damage and/or adnexal adhesions. The factors responsible for tubal disease are diverse and include infections, pelvic surgery, and endometriosis. Pelvic inflammatory disease (PID) represents the link between STD and infertility. In majority of cases, acute PID results from acute bacterial endometritis and salpingitis. Most of the long-term consequences of PID, however, stem from the destruction of normal tubal structure, with or without tubal occlusion [1]. While in developed countries, there has been a decline in the incidence of STD salpingitis and correspondingly in PID by the end of 1980s, a significant rise of STD in Eastern Europe and central Asia, has been documented at the beginning of 1990s [2, 39, 40]. The incidence of infertility following the acute PID depends on various factors and varies from 6% to 60% [1]. In addition, there is a silent, relatively asymptomatic PID, which could be the case in up to 80% of chlamydial infections [41]. Genital infection of *Chlamydia trachomatis* is currently the most common bacterial STD (in 20%–40% of cases) and it coexists with the infection of *Neisseria gonorrheae* in 25%–50% of cases [1]. Manifestation of tubal destruction, however, is dependent also from the ability to activate autoimmune inflammation. During chlamydial infection, similar to most infections, the synthesis of heat shock proteins (HSPs) is strongly upregulated. HSPs are the major antigens and can induce a strong immune response [42]. Because there is a strong amino acid sequence homology between microbial and human HSPs, the induced immune response against microbial HSPs may incite an autoimmune inflammatory reaction in the host, culminating in tubal damage [42, 43].

Endometriosis is characterized by the growth of endometrial tissue outside the uterine cavity. It is a common disorder, affecting 10%–20% of all women of reproductive age [44, 45]. The most frequent clinical presentations of endometriosis include dysmenorrhea, pelvic pain, dyspareunia, infertility, and pelvic mass. In addition to distorted pelvic anatomy, altered peritoneal function, impaired implantation, and endocrine and ovulatory abnormalities, the alterations in humoral and cell-mediated immune system reactions contribute to the endometriosis-associated female infertility [46]. Moreover, endometriosis has been labelled an "autoimmune syndrome". Classical autoimmune diseases, as well as endometriosis, are characterized by polyclonal B-cell activation and production of multiple different autoantibodies [21]. About 40%–60% of patients with endometriosis have elevated autoantibody titers when tested against a panel of autoantigens [47]. They often possess specific antiendometrial antibodies [43, 48, 49], but also AOA, antinuclear autoantibodies (ANA), smooth muscle autoantibodies (SMA), and antiphospholipid antibodies (APA) [23, 50, 51].

Approximately 10%–20% of couples who are unable to conceive are determined to have unexplained infertility [2]. Unexplained infertility is a term applied to an infertile couple whose standard investigations (semen analysis, tubal patency, and laboratory assessment of ovulation) yield normal results. A longer period has been suggested to be required for this group of patients to achieve pregnancy without treatment, as 70% of fertility rate is achieved in two years for the group of unexplained infertility, whereas only nine months are required for the fertile group to achieve the same rate [52]. However, about 20%–30% of these patients remain infertile even after 9 years of attempting to conceive [53]. Therefore, unexplained infertility appears to represent either the lower extreme of normal distribution of fertility, or it arises from a defect in fecundity that cannot be detected by the routine infertility evaluation [2, 54–56]. Dysregulation in immune system reactions with enhanced production of autoantibodies is putative etiologic candidate for this group of patients [32, 57, 58].

Thyroid autoantibodies have been associated with recurrent pregnancy loss, POF, and repeatedly unsuccessful IVF attempts [11, 59, 60]. This is hypothetically explained by the fact that organ-specific autoimmune diseases, like

Table 2: Serum autoantibodies in female infertility and infertility-related diseases.

Patients (N)	Autoantibodies	Methods	Study design	Authors (reference no.)
POF				
POF (45)	AOA 47%* Antioocyte Aab 47%* AOA or anti-oocyte Aab 69%* Anti-LH 6.7% (also AOA positive) AThA 18%* Antiplacental Aab 22%*	ELISA	CC	Luborsky et al. 1990 [30]
POF (45)	AOA 24–60%*	ELISA	CC	Wheatcroft et al. 1994 [68]
POF (48)	Anti-3 beta hydroxysteroid dehydrogenase Aab 21%*	IB, IF, cDNA screening	CC	Arif et al. 1996 [69]
POF (46)	AOA IgG, IgA or IgM 59%—IgG 74.1%, IgA 33.3%, IgM 29.6%*	ELISA	CC	Fénichel et al. 1997 [29]
(A) POF (14) (B) IVF poor responders (29) (C) IVF good responders (14)	FSH blocking IgG: (A) 21.4% (B) 6.9% (C) 85%*	IgG purification, cell culture exposure	CC	Reznik et al. 1998 [70]
POF (30) Unexplained infertility (38)	AOA and AThA 60%* ANA and ACA 16%* AOA 53%* AThA 30%*	EIA	CC	Luborsky et al. 1999 [32]
POS positive for AOA (36)	Anti-FSH (anti-V14D) 94.4%*	ELISA, IB, IF, peptide screening	P	Gobert et al. 2001 [71]
POF (15)	AOA 66.6%* Antizona pellucida Aab 53.3%* TMA 33.3%	IHC	CC	Kelkar et al. 2005 [31]
IVF patients				
IVF poor responders with male infertility or TFI (26)	AOA 77%* Anti-FSH 92%* Anti-LH 65%*	ELISA	CC	Meyer et al. 1990 [72]
IVF failure (80)	AOA 12.5%*	IF	CC	Geva et al. 1999 [62]
IVF failure (17)	1 out of 6 common Aab IgG 82.3%*: ACA 58.8% LA 47.1% AThA 58.8% ANA 58.8% SMA 11.8%	ELISA, IF, PDCA	CC	Putowski et al. 2004 [61]
IVF patients (135): (A) PCOS, endometriosis, unexplained infertility (B) TFI or male infertility	(A) and (B) higher titer of anti-FSH IgG, IgA and IgM* (A) 1 out of 7 common Aab IgG 49%*-ANA 2 preparations, SMA, PCA, ACA, B2-GPI or anti-TPO	ELISA, IF	CC	Haller et al. 2007 [73]
IVF poor responders (16)	Anti-FSH IgA 37.5%* Anti-FSH IgG 31.3%*	ELISA	CC	Haller et al. 2008 [74]
TFI with IVF failure (156)	AEA IgA* (antialpha enolase)	IB, MS	CC	Sarapik et al. 2010 [43]
TFI (21)	Antichlamydial HSP60 antibody titer*	ELISA, IB, IF	CC	Rodgers et al. 2010 [42]
Non-IVF infertility patients				
(A) Unexplained infertility (26) (B) Unexplained abortion (24)	2 APA, 5 antihistone or 4 antipolynucleotide IgG, IgA or IgM (A 88% and B 70.8%)	ELISA	P	Gleicher et al. 1989 [66]
Pregnancy complications (69): (A) Early pregnancy loss (B) Foetal death (C) Preeclampsia	AThA 37.7%*: (A) 37.9%* (B) 40.9%* (C) 33.3%*	ELISA, PDCA, RIA	CC	Mecacci et al. 2000 [11]

Table 2: Continued.

Patients (N)	Autoantibodies	Methods	Study design	Authors (reference no.)
Infertility (108): Menstrual cycle disturbances Anovulation Luteal phase deficiency Unexplained infertility PCOS Endometriosis	1 out of 9 common Aab IgG 40.7%* ANA 13.9%* SMA 27.8%* TMA 1.9%* PCA 0.6% B2-GPI 4.4% ACA 5%	ELISA, IF	CP	Reimand et al. 2001 [25]
Infertility (438): Endometriosis TFI Ovarian dysfunction Male infertility Unexplained infertility	Anti-TPO 14%: 18% in female infertility* 29% in endometriosis*	RIA	CC	Poppe and Velkeniers 2002 [60]
(A) Infertility (178)—PCOS, endometriosis (B) Uncomplicated pregnancy (75)	(A) higher titer of anti-FSH (anti-V14D) IgA* (B) lower titer of anti-FSH (anti-V14D) IgG, IgM*	ELISA	CC	Haller et al. 2005 [75]
	Infertility-related diseases			
Endometriosis (13)	AOA, AEA, anti-theca cell Aab, anti-granulosa cell Aab titers*	IF, PHA	CC	Mathur et al. 1982 [50]
Endometriosis (59)	ANA 28.8%, LA 45.5% (inversely related to disease stage) 1 out of 16 antigens IgG 64.5% 1 out of 16 antigens IgM 45.2%	IF, PDCA	P	Gleicher et al. 1987 [21]
Endometriosis (60)	Anti-α 2HS glycoprotein and antitransferrin titers*	ELISA	CC	Mathur et al. 1999 [49]
PCOS (34)	AOA IgG, IgA or IgM 44%—IgG 27%, IgA 3%, IgM 27%*	ELISA	CC	Fénichel et al. 1999 [19]

* Statistically significant compared to the reference ($P < 0.05$), Aab-autoantibodies, ACA-anticardiolipin autoantibodies, AEA-antiendometrial autoantibodies, ANA-antinuclear autoantibodies, AOA-antiovary autoantibodies, APA-antiphospholipid autoantibodies, AThA-anti-thyroid autoantibodies, B2-GPI-anti-beta 2-glycoprotein I autoantibodies, EIA-enzyme immunoassay, ELISA-enzyme-linked immunosorbent assay, FSH-follicle stimulating hormone, HSP-heat shock protein, IF-immunofluorescence, IB-immunoblot analysis, IHC-immunohistochemistry, IVF in vitro fertilization, LA-lupus anticoagulant, CC-case-control study, CP-cases-population study, LH-luteinizing hormone, MS-mass spectrometry, P-prevalence, PCA-parietal cell autoantibodies, PCOS-polycystic ovary syndrome, PDCA-phospholipid-dependent clotting assay, PHA-passive haemagglutination, POF-premature ovarian failure, RIA-radioimmune assay, SMA-smooth muscle autoantibodies, TFI-tubal factor infertility, TMA-thyroid microsomal autoantibodies, TPO-thyroid peroxidase, V14D-78-93 amino acid immunodominant epitope on FSH.

thyroiditis, may develop secondary to some basic cellular abnormality that directly affects pregnancy outcome [60, 61]. Repeated IVF failure has been associated with increased prevalence of many autoantibodies, including AOA, APA, ANA, SMA, and antisperm antibodies [61, 62]. Therefore, the failure in differentiation of uterine T-cells into T-regulatory cells, as a key determinant of fertility in women has been suggested to be a case in unexplained infertility [58]. Since the prevalence of AOA in unexplained infertility and POF has been detected similar, the unexplained infertility was suggested to represent an early stage of autoimmune POF [32].

The impact of a particular autoantibody on the pathogenesis of infertility is not uniformly understood. ANA could interfere with early implantation of embryo and SMA could alter the fallopian tube function [23]. It is concluded that APA may be involved in uterine vascular modifications affecting implantation processes [63]. Except AOA in ovulatory dysfunctions and disease-specific autoantibodies described in case of endometriosis [43, 48, 49, 64], autoantibodies detected in infertile patients are usually not specific to infertility or to the gynaecological diseases leading to infertility. Furthermore, the number of detectible autoantibodies, in particular, has been proposed to predict the pregnancy rate of IVF treatment [65]. Therefore, some studies suggest lesser importance of specific autoantibodies and stress the key role of overall activation of the immune system in reduced fecundity [12, 65]. Consequently, the autoimmune-associated infertility might be a polyclonal event characterized by immunological defects at the T-cell level which, similarly to classical autoimmune diseases, may manifest itself in abnormal antibody production [66].

3.1. Antiovarian Autoantibodies. Although the presence of AOA immunoglobulin G (IgG) has been documented in different groups of infertile patients (Table 2), there are no epidemiological studies of ovarian autoimmunity. Using an estimated prevalence of autoimmune POF, about 1.1 million

women potentially have ovarian autoimmunity in US, which makes ovarian autoimmunity far more common than Addison's disease, myasthenia gravis, or systemic lupus erythematosus [67].

Some antibodies in the pool of AOA are suggested to associate with a direct action on ovarian tissue, whereas others have no such effects, similar to autoantibodies in other autoimmune diseases [67]. Therefore, it is possible, that several different antigens are involved in ovarian autoimmunity, as both ovarian cellular and zona pellucida/oocyte antibodies have been reported. Antioocyte antibodies were identified already in 1966, and this was also one of the first descriptions of antiovarian autoimmunity [76]. High prevalence of antizona pellucida antibodies have been detected in infertile women, but also in healthy fertile women and even in men [22]. Antibodies to steroid cells (SCA) are more prevalent in POF patients with Addison's disease (73%–87%), but rare in those patients with other autoimmune disease (0%–8%) or in 0%–10% of patients with isolated POF [22]. Steroidogenic enzymes such as 17α-hydroxylase, desmolase (P450-side chain cleavage), 3β-hydroxysteroid dehydrogenase, and 21-hydroxylase have been detected as the molecular targets of SCA [69, 77–80]. The aldehyde dehydrogenase and selenium-binding protein 1 [81], human heat-shock protein 90-beta [82] and antialpha-enolase [83] has recently been identified as unique antigens in antiovarian autoimmunity associated with POF and infertility. Gonadotrophin receptors have been also investigated as a potential autoantibody targets. While antibodies against LH receptor were first identified in 30% of IVF patients and in 50% of infertile patients with endometriosis [68, 84], only few cases of POF patients possessing antibodies to FSH receptor was documented [85]. A later study on FSH receptor blocking ability of these antibodies has allowed questioning the pathophysiological role of anti-FSH receptor antibodies in ovarian failure [70].

Although blocking antibodies are usually considered to interact with receptors, the FSH and LH activity-inhibiting antibodies could also directly recognize gonadotrophins themselves. The presence of anti-FSH and anti-LH antibodies in poor responder IVF patients has been associated with immunization against exogenous gonadotrophins [72]. Until recently, antigonadotrophin antibodies had been described only in POF patients and that with conflicting results. By using different antibody assays, some authors suggest the importance of only anti-LH antibodies [30], while others evidence the association of POF with anti-FSH antibodies [71]. The latter group presented antibodies against β-subunit of FSH in nearly all of the studied AOA-positive POF patients and no anti-LH activity was detected in these samples. Moreover, these antibodies recognized epitopes all over the β-subunit molecule, but a region between amino acids 78 and 93 (V14D) was predominantly recognized in all samples, probably representing the immunodominant epitope [71]. The antibodies detected could readily explain the ovarian failure in POF patients, since this part of the β-subunit of FSH molecule is directly involved in determining the specificity of receptor binding [86]. The ability of anti-FSH to inhibit the function of FSH hormone has been detected in men [87]. We have looked for the information regarding to the presence of anti-FSH IgG, but also IgA and IgM, in different etiologic groups of female infertility, in healthy women and during pregnancy. Pregnancy itself is accompanied with a suppression of the development of new ovulating follicles. This ovulatory quiescence is due to an inhibition of the pituitary during pregnancy, as seen in the decreased response of FSH and LH to GnRH administration [88]. In addition, we have been interested in the etiologic factors for overproducing anti-FSH antibodies of all subtypes in infertile women as well as the putative pathological role of these antibodies on folliculogenesis or on effectiveness of infertility treatment.

4. Follicle Stimulating Hormone

4.1. Regulation of Gonadal Function by FSH. FSH is one of the two pituitary gonadotrophins involved in the regulation of the gonadal function. In females, FSH targets the receptor expressed only on granulosa cells and induces the maturation of ovarian follicle [89]. FSH can influence the development of preantral follicles via paracrine factors [90]. However, growth of antral follicles becomes critically dependent on FSH support, making a preovulatory follicle capable of ovulation and forming a corpus luteum in response to the mid-cycle surge of LH [91]. The role of FSH and its signalling system is central in the normal reproductive function since mutations in human FSH and its receptor are associated with altered ovarian responses to the hormone, resulting in various degrees of reduced reproductive function [92, 93].

4.2. Coding Genes and Molecular Structure of FSH. FSH is a heterodimer, consisting of an α-subunit common to all gonadotrophins (92 amino acids) and a unique β-subunit (111 amino acids in FSH). Glycosylation of the gonadotrophins is important in circulatory persistence, clearance and in bioactivity [94]. In a solvent environment, two FSH molecules form an asymmetric unit in clasped hands-like fashion [86]. The α-subunit carboxy-terminus as well as carbohydrate residues linked to the α-subunit have been implicated in receptor binding and activation [86, 94]. However, there is a cysteine noose, or determinant loop on the β-subunit of FSH molecule (between amino acids 87 and 94), the residues of which (Asp 88, Asp 90, and Asp 93) play a role in determining the specificity of FSH receptor binding [86].

The receptor-binding and hormone specificity determining β-subunit of FSH hormone is coded by *FSHB* gene at the 11p13 [86]. Haplotype analysis has revealed two most prevalent variants of *FSHB* gene—HAP1 and HAP13 [95]. These two core haplotypes have been suggested to be associated with female's fecundity [95], but the association with autoimmunity to FSH through gene expression in central tolerance induction towards FSH had not been studied.

5. Anti-FSH Antibodies Being Primarily Natural Antibodies

We observed the physiological presence of antibodies directed to FSH in a control group of healthy nonpregnant

women, significantly lower values of IgG and IgM but not IgA anti-FSH antibodies during uncomplicated pregnancy [75], and increased levels of these antibodies in infertile women [73, 75]. A total of 233 consecutive women undergoing IVF treatment in Estonia constructed the infertility patient group in our studies. We have demonstrated the production of anti-FSH IgM antibodies associated with peripheral FSH hormone levels. This association was detected among patients with tubal and male factor infertility [73]. The production of autoantibodies can be enhanced if there is elevated level of autoantigen, as elevated FSH levels and AOA in case of premature menopause [19]. Similarly, autoantibodies and insulin levels in pancreatic β cells are correlated [18]. In our study, the level of FSH remained between the reference values for the majority of patients (peripheral level of FSH at the early follicular phase of the menstrual cycle was 8.73 ± 4.69 IU/L). Patients with anti-FSH IgM and FSH correlation had theirs' hormonal level rather lower than in other patients and their infertility was not caused by immune system dysregulation [73]. These results suggest anti-FSH antibodies being primarily the naturally occurring antibodies rather than markers for autoimmunity against FSH hormone. This hypothesis is further supported by the discussion provided by Thomas [96] who concluded that physiological hormone levels remain below a critical threshold for the stimulation of relevant autoimmune reactions [96]. The reason for the correlation between anti-FSH IgM and the level of peripheral hormone is still unknown but could be associated with regulation of FSH bioactivity or with cyclic changes in ovary. The ovulatory process has been compared to a classical local inflammatory reaction and leukocytes have been suggested to participate actively in the cyclic events in the ovary [97–99]. Recently, cumulus and granulosa cells were shown to express cell surface signaling molecules known as pattern recognition receptors acting as sensors of the external environment important for the innate immune system to discriminate self from nonself or altered self antigens [100]. Moreover, a distinct group of mature B-lineage cells, termed B-1 cells are believed to produce IgM natural antibodies, which interact with variety of self determinants and may also cross-react with bacterial antigens [101]. The natural IgMs represent a primitive innate-like layer of adaptive immune system to provide a primary line of defence against systemic infection from viral and bacterial pathogens. There is also evidence that the natural antibodies may contribute to the elimination of autoantigens exposed during tissue damage, for instance, [101].

In addition to the presence in female serum and in ovarian tissue, FSH is also introduced to the genital tract mucosa as a constituent of semen [102]. Female immune system recognizes and reacts to the constituents of semen during insemination, a phenomenon called seminal "priming". Its appropriate activation to induce sperm-prone mucosal tolerance facilitates subsequent pregnancy by sustaining "semiallograft" embryo development [103, 104]. During the process of partner-specific tolerance, cell-mediated and humoral immune reactions are initiated along with the production of antibodies against semen-specific and shared maternal antigens [103], such as FSH [102]. Therefore, the anti-FSH IgA antibodies detected in the female circulation could be alloantibodies initiated by semen. According to this hypothesis, levels of anti-FSH IgA would be, depending on how closely tolerance is induced, correlated with IgA antibodies produced against sperm surface antigens. We studied the correlations among patients with regard to their similarities in immunotolerating conditions in the genital tract: (i) tubal factor infertility group—women with tubal factor infertility and normal semen quality observed in their partners, (ii) male factor infertility group—healthy women and impaired sperm quality observed in their partners, and (iii) combined group of patients—women with endometriosis, PCOS or unexplained infertility and normal semen quality observed in their partners [74]. Among all subtypes of antibodies, anti-FSH IgA and anti-sperm IgA were in correlation in combined group of patients [74]. These results suggest that both detected antibodies share the antigenic origin and we propose anti-FSH IgA represent a natural activation of female immune system in inducing the mucosal tolerance to partner antigens. This idea is supported by the previous study, where anti-FSH-β-chain antibodies were shown to be absent in the sera of children [71].

Somewhat surprisingly, this correlation was only seen in IVF patients with PCOS, endometriosis, and unexplained infertility and not in patients with male factor or tubal factor infertility [74]. The common feature for the former three infertility groups is disturbed regulation of the immune system [19, 24, 25, 57, 58]. Disruptions of the immune system perturb the female's immune response to semen that is necessary for partner-specific tolerance and thereafter elimination of activated clones to prevent autoimmunity during pregnancy [103]. Semen exerts its "tolerance inducing" effect due to immunomodulating factors, most importantly transforming growth factor β_1(TGFβ_1) [105, 106]. Seminal levels of TGFβ_1 correlate with sperm concentration in ejaculate [105], the most decisive criterion for diagnosing male infertility. However, there is some evidence that male factor infertility is not associated with altered TGFβ_1 levels [107]. Although we did not distinguished subgroups of patients with male infertility by sperm parameters, generally their levels of antisperm and anti-FSH antibodies, or correlations between the two, were similar to other patients. Unlike other IVF patients participating in our study, patients with infertility caused by tubal factor do not have disturbances in female immune system regulation or seminal environment. Thus, the diagnosis-restricted correlation of antisperm and anti-FSH IgA cannot be easily explained. However, higher levels of anti-FSH IgA showed an association with the presence of the *HLA-DQB1∗03* allele [73]. In this context, it is interesting to refer to the published associations between the *HLA-DQB1∗03* allele, and the presence of the sperm-immobilizing antibodies in cervical secretions [108]. Higher production of antisperm antibodies has been detected in patients with increased intestinal permeability in bowel inflammatory disease, as a result of immunization against intestinal microbes, which seems to share common antigenic epitopes with spermatozoa [109]. Consequently, the elevated levels of anti-FSH IgA antibodies in IVF patients could be explained by an upregulation of the normal

mucosal immune response. Another possible explanation of the increased anti-FSH IgA in IVF patients could be a deficits in producing antibodies that neutralize anti-FSH immunoglobulins, which has been noted in patients who produce antisperm antibodies [110]. These results together suggest that the elevated values of anti-FSH IgA in IVF patients could represent a failure in mucosal tolerance in the genital tract, which could be genetically determined.

The production of anti-FSH IgG and IgM is decreased during uncomplicated pregnancy [75]. This decrease cannot be easily explained by the general view of a shift towards Th2 cytokines favouring humoral immunity during pregnancy [111]. However, in fact, actual elevations of autoantibodies have been detected in patients with pregnancy loss or recurrent abortion rather than in healthy noncomplicated pregnancy [111, 112]. Therefore, we believe that the development of the FSH-antibodies could reflect some other pregnancy-associated mechanism and that anti-FSH antibodies could be the natural antibodies also in this occasion.

Figure 1(a) summarizes anti-FSH as natural antibodies in healthy women. Humoral immune memory associated with natural antibody-producing B-cells might contribute to the homeostasis of the internal milieu. These cells are also believed to be responsible for autoantigen-mediated clonal selection in the process of initiating autoimmune reactions [101].

6. Increased Production of Anti-FSH Antibodies Contributes to Female Infertility

6.1. Higher Values of Anti-FSH in Infertile Women. We observed that anti-FSH antibodies were predominantly produced in infertile patients compared to healthy female blood donors [73, 75]. As stated earlier, a group of infertile patients from our studies were indicated for IVF, but serum samples were obtained before the administration of exogenous FSH [73]. Thirty-four percent of patients had had at least one previous IVF procedure, but at least three months had passed since the last FSH controlled ovarian hyperstimulation (COH). Furthermore, using stratification by previous IVF procedures, anti-FSH antibody levels were also increased in IVF patients who had never undergone IVF procedures before. The further analysis demonstrated no significant differences in anti-FSH antibody levels between the combined groups of patients with tubal and male factor infertility compared to the women with PCOS, endometriosis, unexplained infertility, and female infertility due to the other causes [73]. These data together suggest that infertility itself, rather than the cause of infertility, could be a predictive factor for the emergence of anti-FSH antibodies, as previously concluded in case of AOA [113]. The intriguing question of what associates the production of anti-FSH antibodies and female infertility stemmed directly from this context.

Female infertility has been shown to be associated with a higher occurrence of autoantibodies [17, 19, 23–25]. Except disease-specific autoantibodies described in case of endometriosis and POF [22, 48, 49], autoantibodies detected in infertile patients [17, 19, 23–25] are usually not specific to infertility or to the gynaecological diseases leading to infertility. Thus, a general immune dysbalance and activation of autoimmune processes are expected to be characteristic for female infertility [12]. We have assessed a potential susceptibility of a patient to autoimmunity by the presence of at least 1 out of 7 common IgG type of autoantibodies in relation to the autoimmunity-prone *HLA-DQB1* alleles [73]. Anti-FSH IgM associated with the production of common autoantibodies and this association was not confounded by the presence of *HLA-DQB1* alleles [73]. Our results along the ones from the literature discussed above indicate that the increased production of anti-FSH IgM could be related to a general propensity to autoimmunity in infertile women.

The female infertility has often been studied in the context of IVF. The follicular puncture performed in IVF, in particular, can induce the production of AOA [17]. In concordance with these data, we showed that the level of anti-FSH IgM was higher in the patients who had undergone previous IVF procedures [73]. The association was revealed among IVF patients who were suffering from PCOS, endometriosis, unexplained infertility, and infertility due to the other causes but not among the women with tubal or male factor infertility. These results encourage us to speculate that repeatedly performed ovarian punctures do not enhance antiovarian autoimmunity unless a patient's infertility is caused by the diseases associated with disturbances in immune regulation [17, 19, 23–25]. However, simply based on the association study performed by us, we cannot substantiate whether the antibodies themselves may cause the need for multiple IVF procedures, or alternatively, the use of IVF procedure *per se* may enhance the production of anti-FSH.

The receptor-binding and hormone specificity determining β-subunit of FSH hormone is coded by *FSHB* gene at the 11p13 [86]. Similarly to insulin gene polymorphisms affecting central tolerance through the level of gene expression in thymus [16], we were looking for an association between the two *FSHB* core haplotypes [95] and autoimmunity against FSH. As we could not detect such relationship [73], we suggest that either these single nucleotide polymorphisms do not affect gene expression in the thymus during central tolerance induction or that *FSHB*-associated autoimmunity to FSH depends on *HLA-DQB1* allelic variants other than those evaluated in our study [73].

The production of anti-FSH IgA is probably related to different factors than those involved in the production of anti-FSH IgM [73]. Anti-FSH IgA were associated with the presence of the *HLA-DQB1∗03* allele [74] but not with the cause of infertility, the history of previous IVF attempts or the presence of other autoantibodies [73]. Therefore, it would be tempting to speculate that anti-FSH IgA could not be autoantibodies but alloantibodies triggered by seminal FSH [102] and originating from mucosal response, as discussed above. The reasons for an increased production of this particular IgA isotype of antibodies in IVF patients, however, remain unclear.

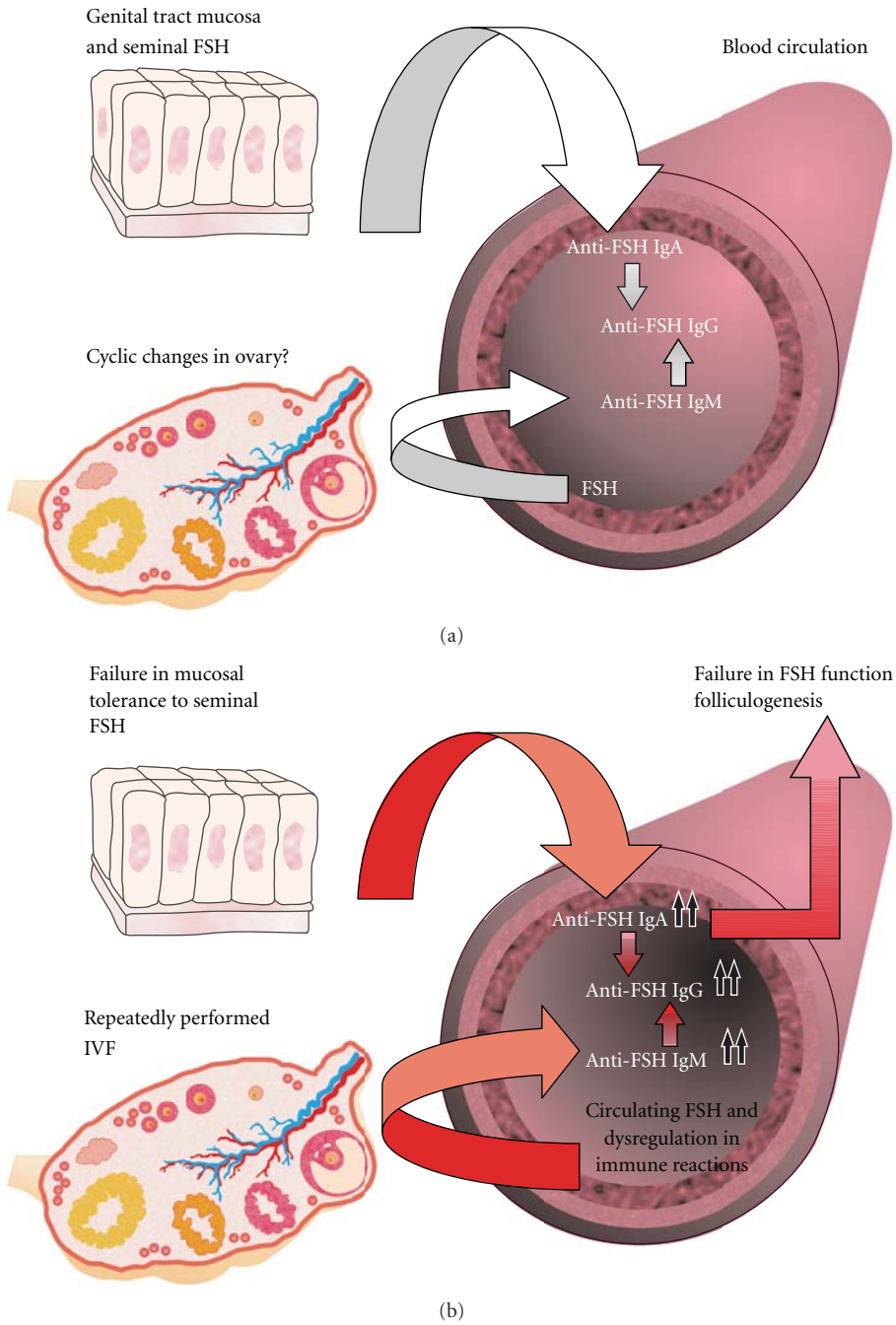

Figure 1: (a) Schematic overview of anti-FSH antibodies in healthy female. Antibodies detected against FSH could be natural antibodies also subjected to pregnancy-associated immune system regulations. Anti-FSH IgA detected in female circulation could be a part of the mucosal response involved in inducing immune tolerance to seminal constituents. Anti-FSH IgM associates with the peripheral level of FSH hormone and possibly contributes along with the mucosal-associated induction of IgA to the production of circulating anti-FSH IgG. (b) Increased production of naturally occurring anti-FSH antibodies in case of female infertility. The production of anti-FSH IgM and IgG antibodies could be related to a general propensity to autoimmunity or to previous IVF treatments. The elevated values of anti-FSH IgA could be explained by a genetically determined failure in mucosal tolerance in the genital tract. Anti-FSH IgG and IgA antibodies, present in sera, accumulate into the preovulatory follicle, where they affect negatively oocyte maturation.

Correlation analysis of anti-FSH antibody values among healthy controls showed that the levels of anti-FSH IgM and IgA correlated both with the values of anti-FSH IgG [73]. There is some indirect evidence that anti-FSH IgG antibodies may, however, further worsen female fecundity by reducing the FSH functionality [70, 72]. These data lead us to investigate the effect of anti-FSH antibodies on folliculogenesis and developing infertility in women.

6.2. Effect of Serum Anti-FSH on Folliculogenesis. IVF has become a promising treatment for various causes of infertility. However, the success of attaining pregnancy following IVF depends on the effectiveness of COH. Serum levels of anti-FSH IgG and IgA, but not IgM antibodies at the day of oocyte retrieval, were in linear association with poorer outcome of COH [114]. The outcome of COH was defined by the duration of FSH stimulation or the total FSH required attaining an adequate response, the number of follicles punctured or oocytes obtained after COH, the number of mature oocytes or embryos, and the amount of FSH required per all of these parameters. The role of anti-FSH antibodies revealed in our study was quite remarkable. For example, our data suggest that a unit of difference in anti-FSH IgG was associated with a 220.6 IU increase in FSH needed for one zygote, while the mean amount of FSH per zygote was only 443.8 ± 401.2 IU. Furthermore, the cutoff value of >1.0 for anti-FSH IgA and IgG was calculated to be implicated to poor ovarian response (≤3 oocytes) [114]. Series of dilutions of mouse anti-human-FSH monoclonal IgG antibody were used in ELISA test to create a concentration curve and to predict serum anti-FSH IgG antibody concentration. According to the curve, the levels of anti-FSH IgG > 1.0 was presumed to correspond to the antibody levels higher than 0.5–0.6 mg/L and could, therefore, represent 0.004% of expected amount of total IgG (8–17 g/L) in peripheral blood. The same or even slightly lower levels of blocking and stimulating serum TSH-receptor autoantibodies has been demonstrated previously in patients with Graves' disease and in autoimmune hypothyroidism [115]. Since anti-FSH antibodies are often detected in patients with AOA [71, 116] our results may simply reflect an impaired ovarian function due to ovarian autoimmunity. The association between antigonadotrophin [72] or AOA [67] IgG in the sera at oocyte retrieval and poor ovarian response to the FSH stimulation has been shown previously.

In addition to reflecting ovarian autoimmunity, anti-FSH antibodies may impair the function of exogenous or endogenous FSH. For example, anti-FSH could form immune complexes with FSH and induce its clearance, as recently shown for creatine kinase in patients with corresponding antibodies [117]. Also, anti-FSH could interrupt the binding of FSH to its receptor. This hypothesis is supported by our data suggesting anti-FSH antibodies in sera correlated with antibodies directed against the 78–93 amino acid region of the β-chain of the human FSH [71, 75], the domain that determines FSH receptor binding specificity [86]. On the other hand, the study of *in vitro* FSH-blocking ability of anti-FSH IgG in women with good IVF response [70] suggested that anti-FSH antibodies may be nonpathogenic. However, this study did not specify which FSH epitopes were bound by the pool of anti-FSH antibodies.

Although the pathophysiology of anti-FSH in association with poor ovarian response is still unclear, the importance of these antibodies is noteworthy. Woman's age and her ovarian volume and the number of follicles counted at the early follicular phase of her spontaneous menstrual cycle were significant clinical parameters predicting the outcome of COH [114], as also demonstrated by others [118]. Yet, anti-FSH antibodies could represent an additional importance to the clinical parameters like age, follicle number, or ovarian volume in predicting the outcome of COH. Furthermore, if the influence of anti-FSH on the ovarian response is revealed in the IVF patients (where supraphysiological amounts of FSH were administered to stimulate folliculogenesis), the importance of those antibodies in unstimulated spontaneous folliculogenesis might be substantial.

6.3. Changes in Serum Levels of Anti-FSH during COH in Relation to Follicular Fluid. Serum levels of anti-FSH IgG and IgA, but not IgM antibodies, decreased following COH, conducted with GnRH antagonist protocol [114]. Although interpretation of these results is not straightforward, we believe the decrease in anti-FSH antibody levels could partly be explained by the supraphysiological levels of immunosuppressive progesterone and testosterone [114, 119, 120] produced in COH. This hypothesis is supported by our previous data suggesting an overall decrease in the number of common IgG autoantibodies during COH [57]. Additionally, anti-FSH antibodies could form immune complexes with administered recombinant FSH or with endogenous FSH (produced in pituitary prior to administration of GnRH antagonists), resulting in the decrease in antibody levels. However, the levels of anti-FSH IgM remain unchanged after COH [114]. As IgM antibodies also form immune complexes, the reactivation of the immune system towards novel epitopes on the FSH molecule and the production of anti-FSH IgM during COH might be speculated. As well, immunization against exogenous gonadotrophins has also been previously suggested [72]. This hypothesis is further supported by our findings and that found from the literature that an increase in IgM type of anti-FSH [73] and AOA [17, 32, 73, 121] associated with repeated IVF procedures. However, it was also reported that AOA were initiated by ovarian puncture rather than administered FSH [17]. Additionally, circulating anti-FSH could pass into the follicular fluid during follicle maturation; however, this decrease would hardly be detectable in sera by current laboratory tests.

The charge- and size-selective ovarian blood-follicle barrier is open for IgG to pass into the follicular fluid [122] and the concentration of total IgG and IgA in follicular fluid as well as in blood should be equivalent [123]. We have measured the presence of anti-FSH IgG, IgA and IgM in negligible amounts in follicular fluid [114]. The level of anti-FSH IgA also correlated with the level of same antibody in peripheral blood [114]. However, anti-FSH IgG seemed to accumulate into the growing follicle, since the concentration of follicular anti-FSH IgG associated positively with the diameter of a follicle, reflecting the maturity of a follicle [114]. The increase in follicular anti-FSH IgG with the growth of the follicle is not a simple reflection of anti-FSH IgG serum levels, as serum anti-FSH IgG levels significantly decreased during COH [114]. Logically, follicular anti-FSH IgG levels correlated with the amounts of recombinant FSH used for COH and FSH levels measured in the follicle [114]. The level of follicular FSH increases while the follicle grows

[124, 125], and expectedly, follicular FSH correlates with the amount of FSH administered exogenously [67, 114]. Thus, anti-FSH IgG could diffuse along with the antigen to the follicular fluid during the COH. Although anti-FSH IgA and IgM were detected in the follicle, levels of these antibodies were not associated with follicle diameter [114], which is in agreement with other authors [126]. In addition, anti-FSH IgM levels in the follicle were very low compared to serum antibody levels [114], in concordance with that reported by Clarke and coworkers [123], where total IgM in the follicle represented approximately 10% of its plasma concentration [123]. Figure 1(b) summarizes our studies on anti-FSH antibodies in cases of female infertility. These results emphasize the need for further research to elucidate the clinical relevance of anti-FSH antibodies in the spontaneous menstrual cycles.

Finally, low-dose prednisolone therapy has improved pregnancy rate in patients with recurrent IVF failure [62, 67, 127] and in non-IVF patients [128]. Different treatment regimes of oral prednisolone has been suggested, such as 10 mg/d during one month prior to the COH [62], 0.5 mg/kg/d starting from the beginning of COH until the end of 1st trimester of pregnancy, and followed by lowering the dose thereafter [127], or 10 mg/d in the 1st week, 5 mg/d in the 2nd week, 2.5 mg/d in the 3rd week, and 2.5 mg/d 3 times a week during the last (4th) week before intrauterine insemination [128]. However, considering the time duration of ovarian folliculogenesis, the treatment should start at least 1-2 months before COH [67]. Most benefit of immunosuppressive treatment can gain infertile patients who represent antiovarian autoimmunity [129]. Testing serum anti-FSH antibodies could help infertility treatment specialists to identify those patients.

7. Conclusions

Female fertility can be affected by diseases or dysfunctions of reproductive tract, neuroendocrine system, and immune system. Reproductive autoimmune failure can be associated with overall activation of immune system or with immune system reactions specifically directed against ovarian antigens. Antiovarian autoantibodies are mostly directed against β-subunit of follicle stimulating hormone (anti-FSH). Anti-FSH could be natural antibodies. Anti-FSH IgA detected in female circulation could be a part of the mucosal response involved in inducing immunotolerance to seminal constituents. Anti-FSH IgM associates with the peripheral level of FSH hormone and contributes along with the mucosal-associated induction of IgA to the production of circulating anti-FSH IgG. Additionally, higher production of anti-FSH antibodies could contribute to female infertility. The induced production of anti-FSH IgM antibodies could be related to a general propensity to autoimmunity or to previous IVF treatments. The elevated values of anti-FSH IgA could indicate genetically determined failure in mucosal tolerance in the genital tract. Serum IgG and IgA anti-FSH antibodies, measured at the day of oocyte retrieval, predict the outcome of ovarian stimulation, additionally to that observed with age and other clinical parameters characterizing the ovarian reserve. A population of anti-FSH antibodies which are produced against 78–93 epitope on the β-chain might modulate the recognition and binding of FSH to its receptor and might, therefore, have a pathological influence on ovarian function. We have also demonstrated that anti-FSH IgG, IgA, and traces of IgM antibodies were detectable in the follicular fluid and that anti-FSH IgG antibodies accumulated into the preovulatory follicle. Immunosuppressive treatment could improve the pregnancy rate in anti-FSH seropositive infertile patients.

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Review Article

Immune Aspects and Myometrial Actions of Progesterone and CRH in Labor

Nikolaos Vrachnis,[1] Fotodotis M. Malamas,[2] Stavros Sifakis,[3] Panayiotis Tsikouras,[4] and Zoe Iliodromiti[1]

[1] *2nd Department of Obstetrics and Gynecology, University of Athens Medical School, Aretaieio Hospital, 11528 Athens, Greece*
[2] *1st Department of Obstetrics and Gynecology, University of Athens Medical School, Alexandra Hospital, 11528 Athens, Greece*
[3] *Department of Obstetrics and Gynecology, University Hospital of Heraklion, 71110 Heraklion, Crete, Greece*
[4] *Department of Obstetrics and Gynecology, University Hospital of Alexandroupolis, Democritus University of Thrace, 68100 Alexandroupoli, Greece*

Correspondence should be addressed to Nikolaos Vrachnis, nvrachnis@med.uoa.gr

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Progesterone and corticotropin-releasing hormone (CRH) have a critical role in pregnancy and labor, as changes related to these hormones are crucial for the transition from myometrial quiescence to contractility. The mechanisms related to their effect differ between humans and other species, thus, despite extensive research, many questions remain to be answered regarding their mediation in human labor. Immune responses to progesterone and CRH are important for labor. Progesterone acts as an immunomodulator which controls many immune actions during pregnancy, and its withdrawal releases the inhibitory action on inflammatory pathways. In humans, a "functional" progesterone withdrawal occurs with onset of labor through changes in progesterone metabolism, progesterone receptors, and other molecules that either facilitate or antagonize progesterone function. Placental CRH acts on the fetal pituitary-adrenal axis to stimulate adrenal production of androgens and cortisol and also acts directly on myometrial cells via its receptors. CRH also affects inflammatory signals and vice versa. Interactions between progesterone and CRH additionally occur during labor. We describe the role of these two hormones in human myometrium and their interactions with the immune system during labor.

1. Introduction

Mechanical and endocrine mechanisms, immune system responses with inflammatory signals, and release of cytokines, prostaglandins, and oxytocin contribute among others to the transition from myometrial quiescence to labor initiation. The role of these agents has been extensively studied in many animal models, although differences exist between species, and therefore these observations do not always apply to humans [1]. Progesterone and corticotropin-releasing hormone (CRH) are both among the most important mediators of labor. Their role has been studied in many different tissues and organs during pregnancy and labor [2]. Progesterone withdrawal occurs towards the onset of labor through many mechanisms. Placental CRH acts on the fetal pituitary-adrenal axis and directly on myometrial cells to facilitate labor.

Apart from changes in the endocrine milieu, immune system responses are also vital for labor. During the onset of labor immune cells such as neutrophils, macrophages and T cells invade into cell membranes, decidua, cervix, and myometrium as they are attracted by local chemokines [3, 4]. Characterization of the human myometrial transcriptome and comprehension of the changes in gene expression during spontaneous labor at term have considerably elucidated the association between spontaneous labor and biological processes such as inflammatory response, chemotaxis, and immune response, as well as molecular functions like cytokine and chemokine activity and chemokine receptor binding. Among the overexpressed genes were interleukin

8 (Il-8), Il-6, monocyte chemotactic protein-1 (MCP-1), leukocyte immunoglobulin-like receptor, subfamily A member 5 (LILRA5), chemokine C-C motif ligand 6 (CXCL6), nuclear factor of kappa-light chain gene enhancer in B-cells inhibitor zeta (NFKBIZ), and suppressor of cytokine signaling 3 (SOCS3). Software for pathway analysis of microarrays and gene expression data revealed that the pathways involved in inflammatory response were enriched during spontaneous labor at term [5].

The increased expression of cytokines in the myometrium results in increased contractility. Proinflammatory cytokines contribute to the onset of labor irrespectively of the presence of inflammation. Interleukin 1-β (IL-1β), IL-6, and IL-8 trigger the transcription of genes through activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [6]. IL-6 is considered to be mainly a proinflammatory cytokine [7, 8], but may possibly also have anti-inflammatory properties and its upregulation in parallel with IL-1β and tumor necrosis factor-alpha (TNF-α) may contribute to a balance between pro- and anti-inflammatory cytokines [9]. Lipoxin A4 is an anti-inflammatory compound that may moderate inflammatory response and modulate inflammatory events taking place in the myometrium during parturition [10]. During labor, myometrial cells are connected by gap junctions that are created by multimers of connexin 43 for the achievement of coordinated and synchronous myometrial contractions, while progestins repress connexin 43 gene expression in myometrial cells [11–13]. Moreover, oxytocin receptors (OTRs) in myometrium increase during labor, and oxytocin promotes myometrial contractility by increasing the intracellular Ca^{2+} ions and the production of prostaglandins [14–16]. In uterine tissues proinflammatory cytokines such as IL-1β and TNF increase cyclooxygenase-2 (COX-2) expression, OTRs, Prostaglandin H Synthase type 2 (PGHS-2), and connexin-43 [6]. In this study we review some of the most recent advances in scientific knowledge concerning progesterone and CRH function and interactions in human labor, placing particular focus on the effect on myometrial cells. We also discuss the immune system responses to these hormones.

2. Progesterone Withdrawal in Labor

According to the "progesterone block" hypothesis, proposed by Arpad Csapo, progesterone blocks myometrial contraction and maintains pregnancy, while its withdrawal transforms the myometrium to the laboring state [17]. In rats and other animals the initiation of labor coincides with a decline in progesterone serum concentration. However, in humans progesterone levels remain high throughout pregnancy and during labor [1]. This has led to the hypothesis of a "functional" progesterone withdrawal that may occur through mechanisms such as progesterone metabolism into inactive forms, expression of different progesterone receptor isoforms, altered expression of molecules that act as progesterone coregulators, and finally the antagonism of NF-κB [18] with progesterone (Figure 1).

2.1. Progesterone Metabolism. In mice, the catabolism of progesterone to the inactive C21-steroid 5α dihydroprogesterone by the enzyme steroid 5α-reductase facilitates cervical ripening, while mice deficient in this enzyme fail to deliver at term despite normal uterine contractions and progesterone withdrawal in blood [19]. In mice with a targeted disruption of 20α-hydroxysteroid dehydrogenase (20α-HSD) gene (which converts progesterone to the biologically inactive metabolite 20α-dihydroprogesterone), the mean duration of pregnancy is significantly prolonged compared to the controls [20]. In cultured rat ovarian granulose cells IL-1β stimulates 20α-hydroxysteroid dehydrogenase activity [21]; however it is unknown if such an effect may also take place in myometrium during labor. In humans, experiments with endocervical cells of women in labor show that, during cervical ripening and parturition, 17β-hydproxysteroid dehydrogenase type 2 activity decreases, resulting in increased 20α-hydroxyprogesterone and estradiol levels because of decreased conversion of 20α hydroxyrogesterone to progesterone and decreased conversion of estradiol to estrone. These changes result in inactivation of progesterone responses [22].

2.2. Altered Expression of Progesterone Receptors. The role of progesterone receptors in human pregnancy has been recently extensively reviewed [23]. In humans, there are two major isoforms of progesterone receptor, PR-A and PR-B, which belong to the nuclear receptor superfamily, as well as many other isoforms which have so far given evidence of being less significant. The expression of the various isoforms may contribute to the functional withdrawal of progesterone during labor. In human myometrial cells, the ratio of PR-A:PR-B mRNA increases 2- to 3-fold compared with the nonlaboring state, mainly due to overexpression of PR-A. This change induces a "functional estrogen activation" through increased estrogen receptor α (ERα) expression [24]. PR-A may also suppress the transcriptional activity of PR-B, which is the main receptor for the nuclear signal transduction of progesterone [25, 26]. Apart from myometrial contractions, the functional progesterone withdrawal due to the altered expression of PR-A, PR-B isoforms may also contribute to the cervical changes during labor [27, 28].

The binding of progesterone to PR-C, which is a soluble form of the receptor, may sequester available progesterone away from PR-B and thereby diminish its biological effect [18]. In another study of women in labor, PR-C protein levels were increased in cytoplasmic fractions of fundal myometrial cells, but not in cells of the lower uterine segment, while PR-B protein levels increased only in the laboring fundal endometrium. In the same study PR-A could not be detected despite the presence of PR-A mRNA. This paradoxical increase in PR-B protein may be the result of the associated reduced progesterone activity. In human telomerase reverse transcriptase- (hTERT-) immortalized human myometrial cells, PR-C compromises PR-B transactivation. The upregulation of PR-B and PR-C expression was associated with activation of NF-κB, while upregulation of NF-κB was observed in pregnant mouse uterus, coinciding with

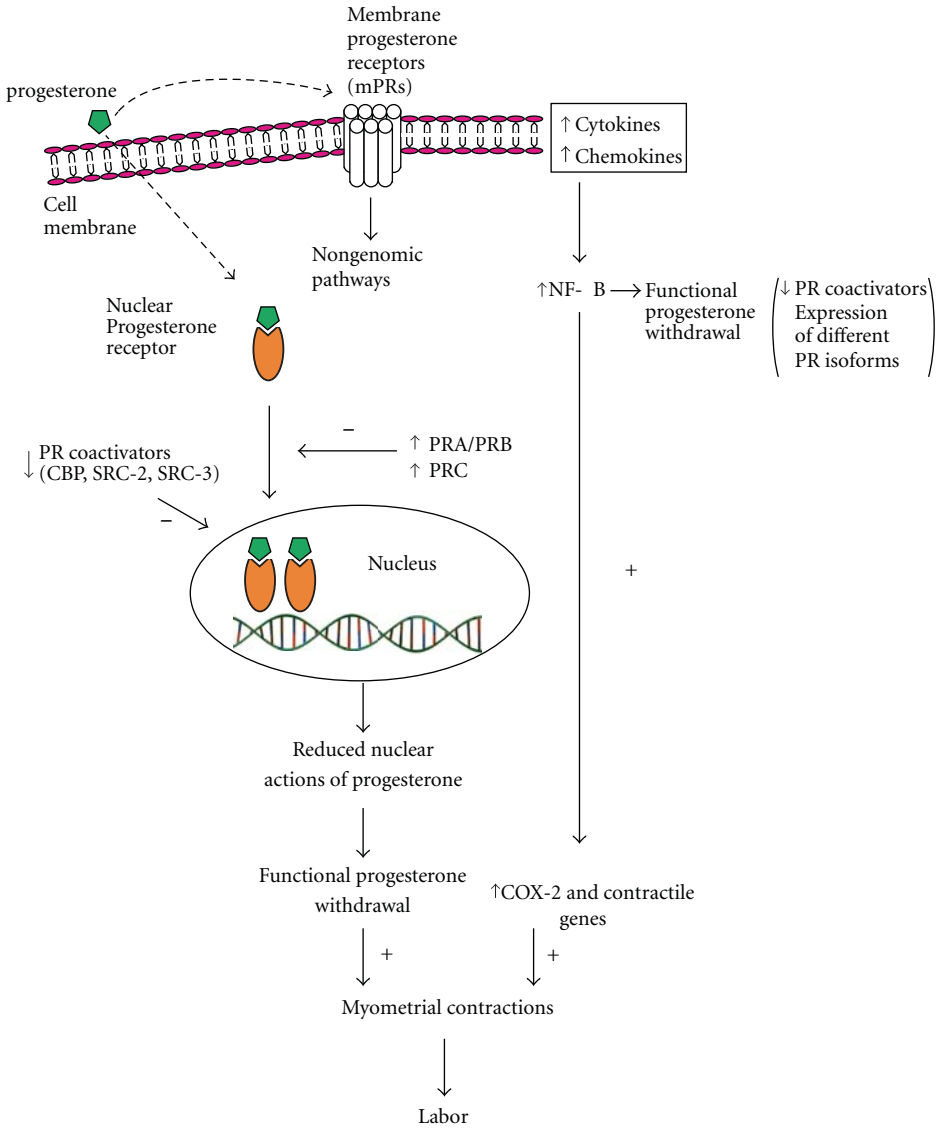

Figure 1: Mechanisms of functional progesterone withdrawal for the initiation of parturition. Progesterone binds to its nuclear receptor PR and activates genomic pathways that maintain uterine quiescence. Functional progesterone withdrawal occurs through many mechanisms: (a) the expression of different PR isoforms (increased PR-A:PR-B ratio and the expression of PR-C), (b) the decreased expression of PR coactivators, such as CBP, SRC-2, SRC-3, (c) binding of progesterone to membrane progesterone receptors (mPR) which activate nongenomic pathways, and (d) immune factors such as cytokines and chemokines activating NF-κB, that in turn lead to functional progesterone withdrawal. NF-κB increases the expression of COX-2 and contractile genes. Progesterone withdrawal together with increased expression of COX-2 and contractile genes results in increased myometrial contractions, which is a vital component for the initiation of labor. PR: progesterone receptor, CBP: CREB-binding protein, SRC: steroid receptor coactivators, NF-κB: nuclear factor kappa-light-chain enhancer of activated B cells, mPRs: membrane progesterone receptors, COX-2: cyclooxygenase-2.

PR isoform expression. In addition, treatment of myometrial cells with the cytokine IL-1β further induces NF-κB activation and concomitant increase in PR expression, which suggests that labor-associated changes in myometrium are associated with uterine inflammatory pathways [29].

A number of other progesterone receptors (mPR-α, mPR-β, and mPR-γ) have been identified which are believed to be structurally related to the G-protein complex. mPR-α and mPR-β receptors were found to be expressed in human pregnant myometrial cells, and progesterone activation of these receptors may induce nongenomic actions, resulting in inhibition of adenyl cyclase, phosphorylation of myosin light chains, and contractions of human myometrial cells in labor. It is suggested that mPR at the end of pregnancy downregulates steroid receptor coactivator-2 (SRC-2), which in combination with an altered PR-B:PR-A ratio may lead to decreased transcriptional activation of PR-B [30]. Controversy exists regarding the function of mPRs on cell membrane, some authors supporting the view that the isoforms of these receptors are intracellular and reside primarily

on the endoplasmic reticulum, while progesterone does not induce any G protein-dependent signaling of mPRs [31].

2.3. Altered Expression of PR Coregulators. PRs interact with coregulators that either increase (coactivators) or decrease (corepressors) their transcriptional activity. These molecules include among others the CREB-binding protein (CBP) and the steroid receptor coactivators SRC-1, SRC-2, and SRC-3. In fundal myometrial cells of women in labor, the mRNA levels of SRC-2, SRC-3, and CBP are decreased compared to the nonlabor samples. Nuclear levels of CBP, SRC-2, and SRC-3 proteins were also reduced in fundal myometrium during labor. In the mouse uterus in labor, levels of SRC-1, SRC-3, and CBP were also reduced. The reduced levels of these coactivators were related to decreased acetylated histone H3 in both human and mouse, suggesting the closing of chromatin structure and the reduced expression of PR-responsive genes as a possible mechanism for the initiation of labor [32]. Histone acetylation/deacetylation can also affect NF-κB activity and proinflammatory gene expression (e.g., IL-6, IL-8, COX-2, and RANTES), while histone deacetylase inhibitors exert anti-inflammatory effects in myometrium which may favor uterine quiescence [33].

p54nrb (non-POU-domain-containing, octamer binding protein) is another molecule that acts as a PR transcriptional corepressor in vitro and which decreases in rat myometrium at term pregnancies. Its decreased expression at the time of labor may contribute to the increased expression of labor-associated genes [34].

2.4. Antagonistic Action of NF-κB. An antagonism between NF-κB and progesterone exists in reproductive tissues during labor. NF-κB belongs to the superclass 4 of transcriptional factors and is involved in the synthesis of many proinflammatory mediators such as cytokines and COX-2. Inflammation is a critical trigger for the initiation of term and preterm labor [35], and NF-κB as a transcriptional factor is implicated in proinflammatory stimuli signaling. Proinflammatory cytokines such as IL-1β and TNF-α, as well as lipopolysaccharides (LPSs), can stimulate NF-κB activity in the uterus. Additionally, the promoter region of cytokines genes such as IL-1β, IL-8, and IL-6 contains NF-κB recognition elements, and NF-κB promotes the synthesis of cytokines [36].

NF-κB also stimulates prostaglandin synthesis, regulates matrix metalloproteinase expression, connexin-43 (a gap junction protein), oxytocin receptor, and may express inhibitory PR isoforms or increase progesterone metabolism [18, 36]. In lower segment fibroblasts and in amnion cells IL-1β induces the synthesis of NF-κB regulated genes, while progesterone attenuates these effects [37]. Progesterone exhibits anti-inflammatory properties that maintain uterine quiescence. MCP-1 is a chemokine which is produced by decidual, endometrial, myometrial, and trophoblast cells. It is increased in laboring pregnant myometrium prior to the onset of labor and attracts leukocytes from the circulation to the myometrium. MCP-1 is also increased in cultured myometrial cells in response to IL-1β. Experimentally, in choriodecidual cells MCP-1 expression is suppressed by progesterone, while, by contrast, it is stimulated by NF-κB [38–40].

Antagonism between nuclear factors such as NF-κB and PR for nuclear cofactors is another proposed mechanism of functional progesterone withdrawal. During labor, the level of NF-κB changes in the intracellular environment, and its activity is regulated by interactions with the nuclear cofactors that increase gene expression, such as CBP/p300. The antagonism between NF-κB and PR for CBP may contribute to progesterone withdrawal and expression of labor-promoting genes [41].

2.5. Progesterone as an Immunomodulator in Pregnancy and the Antagonism with Cytokines. Progesterone acts as an immunomodulator that interacts with the immune system and exerts anti-inflammatory effects throughout pregnancy. Progesterone inhibits the activity of dendritic cells (DCs) that generate proinflammatory responses and favor the induction of tolerogenic DCs. It also controls the activity of natural killer (NK) cells and the differentiation of T cells into T-helper cell type 2 (Th2) like clones. The Th2 phenotype induced by progesterone is a prerequisite for the maintenance of pregnancy [42, 43].

Progesterone-induced blocking factor (PIBF) is a protein which is released from lymphocytes in response to progesterone, mediates the immunological effects, and induces the production of Th2 dominant cytokines like IL-3, IL-4, and IL-10 [44]. The functional progesterone withdrawal and the attenuation of progesterone's effects may contribute, among other factors, to the swift from a Th2 to a Th1 dominant effect towards labor. Inflammatory response involves Toll-like receptors, and progesterone modulates the expression of these receptors in mouse cervix and placenta [45].

Cytokines such as IL-1β and TNF-α increase PGHS-2 and therefore the synthesis of prostaglandins (PG), while they downregulate prostaglandin 15-hydroxy dehydrogenase (PGDH), the enzyme that converts PGs into inactive metabolites. On the contrary, progesterone promotes PGDH expression [6]. Progesterone also downregulates prostaglandin F2-alpha receptor, which is increased by IL-1β [46]. Via the use of expression microarray and quantitative reverse transcriptase PCR it has been shown that in human myometrial cells medroxyprogesterone acetate, a synthetic progestogen, affects genes involved in inflammatory response and cytokine activity. Among the downregulated genes of the study were those of IL-1β, Il-6, Il-11, Il-24, COX-2, and connexin 43 [47].

Clinically, women with threatened preterm labor have significantly lower serum concentrations of PIBF and IL-10 and significant higher serum concentrations of the proinflammatory cytokines, IL-6 and interferon-γ (IFN-γ), compared with healthy pregnant women of the control group [48].

2.6. The Effect of Progesterone Withdrawal on ZEB 1, 2 and miRNAs. A novel pathway for progesterone withdrawal and increased expression of contraction-associated genes has

recently been described based on experiments in mice and human endometrial cells. The levels of micro-RNAs (mi-RNAs), a class of posttranscriptional regulators that belong to the mi-RNA-200 family, increase with advanced gestation, as do the levels of connexin-43 and oxytocin receptor, while the levels of ZEB1 and ZEB2 (zinc finger E-box binding homeobox proteins 1 and 2) repressor transcriptional factor decline. ZEB1 and ZEB2 inhibit the expression of connexin-43 and oxytocin receptor and block the oxytocin-induced contraction of myometrial cells. ZEB1 and ZEB2 also inhibit the expression of the miRNA-200 family, which in turn downregulate ZEB1, ZEB2. Progesterone increases the levels of ZEB1, and thus progesterone withdrawal reverses the inhibitory effect of ZEB on mi-RNAs. As mi-RNAs increase, they cause repression of ZEB and thereby promote the synthesis of connexin-43 and oxytocin receptor, thus facilitating labor [49].

In summary, all the above described mechanisms may collectively impair progesterone regulation of gene expression, and this functional progesterone withdrawal promotes uterine contractility, effecting labor.

2.7. Clinical Applications of Progesterone in Human Labor. Progesterone has clinical applications with regard to either the initiation of labor or the arrest of preterm labor. In women, treatment with antiprogestins induces labor and delivery, but the most significant applications concern prevention of preterm birth. Numerous studies and meta-analyses have been conducted to evaluate the efficacy of progesterone in reducing the incidence and complications of preterm labor. Progestational agents may reduce the number of preterm labor and their complications in certain cases, but the optimal route of administration, the optimal dose, and the frequency of administration have not been clearly defined [50–54].

3. Corticotropin-Releasing Hormone Function on Myometrial Contractility

CRH levels in plasma rise exponentially in human pregnancy. However, this increase is more rapid in women who deliver preterm and slower in women who deliver postterm, compared to women who deliver at term. This rise begins early in pregnancy (16–20 weeks) and provides evidence that placenta acts through CRH as a "clock" that controls the length of pregnancy. Moreover, preterm birth may not be just the result of an event around the time of delivery but a process that begins from early in pregnancy [55].

3.1. Functions of Placental CRH. The rise of CRH is associated with a concomitant fall in CRH binding protein (CRHBP) in late pregnancy. The binding of CRH to CRHBP makes it biologically inactive and removes it from circulation. The decrease in CRHBP during the final three weeks of pregnancy results in higher concentrations of active CRH in circulation that contribute to the onset of labor [55, 56]. In both term and preterm labor, the expression of CRH mRNA from the placenta is increased, with this increase being higher in preterm deliveries. However, placental CRHBP mRNA expression does not change despite the fact that CRHBP decreases in maternal circulation before labor. This leads to the hypothesis that another source of CRHBP exists, such as a fetal source that may be responsible for this decrease [55, 57].

However, despite the fact that the levels of CRH in maternal circulation rise by as much as 1000 times compared to the nonpregnant state, only a mild hypercortisolism occurs, mainly due to estrogen-induced production of cortisol-binding protein, and the maternal axis continues its function. Thus the functional target of placental CRH is not the maternal pituitary adrenal axis but the fetal pituitary adrenal axis [56]. Placental CRH stimulates adrenocorticotropic hormone (ACTH) production from the fetal pituitary. ACTH stimulates fetal adrenals to produce dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulphate (DHEA-S), and cortisol. ACTH is also produced in the placenta through paracrine mechanisms. Placental CRH also exerts a direct effect by stimulating fetal adrenal zone cells (Figure 2). Fetal adrenal DHEA is metabolized to estrogens in the placenta that favor parturition [2, 58, 59]. The produced cortisol exerts a stimulatory effect on the placenta to further produce CRH, thus a positive loop is established that causes placental CRH to rise exponentially as pregnancy advances. Estrogens, progesterone, and nitric oxide inhibit CRH production, while a number of neuropeptides exert a stimulatory effect [2]. Cytokines influence CRH production. IL-1β and TNF as well as lipopolysaccharides activate the hypothalamic-pituitary-adrenal axis and induce CRH production, while CRH regulates IL-1β, IL-6, and lipopolysaccharides production by immune cells [60]. Cytokines also influence the metabolism of cortisol. The enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) converts cortisol to the inactive metabolite cortisone. Cytokines such as IL-1β, IL-6, and TNF-α inhibit human placenta 11β-HSD type 2 isoform, and this could result in increased cortisol levels and therefore increased CRH production by the placenta [6].

Cortisol produced by fetal adrenals acts on fetal lungs and produce surfactant protein A (SP-A) that activates inflammatory signals in the uterus, which consequently enhance myometrial contractility [18]. SP-A signals via Toll-like receptors. These are membrane-spanning receptors that act as key regulators in immune system responses [61]. SP-A exerts its action via activation of NF-κB in many cells. In the mouse, SP-A increases the production of IL-1β and NF-κB by amniotic fluid macrophages and causes migration of macrophages in the uterus, resulting in the activation of the inflammatory cascade [62].

CRH may also facilitate the expression of contraction-associated genes. In myometrial smooth muscle cells from nonpregnant women, CRH enhances connexin-43 mRNA and protein expression through nuclear transcription factor activator protein 1 (AP-1) activation and upregulation of c-Fos expression, which is an AP-1 subunit. This is accomplished in a positive CRH-dose-dependent manner. As a result, in late pregnancy the high levels of CRH possibly cause an associated increased expression of connexin-43 that promote myometrial contractions [63]. AP-1 also increases gene expression in response to many other stimuli, including

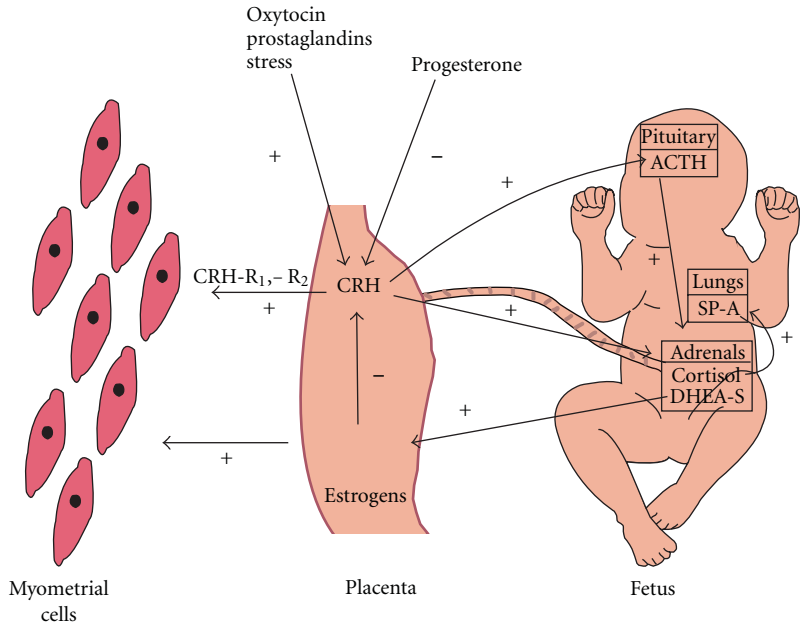

Figure 2: The role of CRH in human labor. CRH is produced in the placenta in response to stimuli that either increase (oxytocin, PG, stress) or decrease (progesterone) its production. CRH acts on fetal pituitary to increase ACTH production. ACTH and CRH act on fetal adrenals that produce DHEA-S and cortisol. DHEA-S is metabolized in the placenta to estrogens that increase myometrial contractions and facilitate labor. Cortisol acts on fetal lungs which produce SP-A, which also increases uterine contractility. Furthermore, placental CRH acts directly on human myometrial cells via its receptors CRH-R1, -R2, to facilitate the transition from uterine quiescence to myometrial contractions during labor. CRH: corticotrophin-releasing hormone, PG: prostaglandins, ACTH: adrenocorticotropic hormone, DHEA-S: dehydroepiandrosterone sulphate, SP-A: surfactant protein A.

cytokines [64]. CRH may also increase the myometrial response to prostaglandin F2α and therefore modulate the onset of labor [65].

3.2. The Role of Myometrial CRH Receptor Isoforms in Labor. CRH exerts its actions through activation of specific receptors, termed CRH-R1 and CRH-R2. These receptors belong to the family of seven transmembrane (7TMD) G protein coupled receptors (GPCRs). CRH receptors are expressed in several tissues in pregnancy. Both CRH-R1 and CRH-R2 and their variants have been detected in human myometrium, and it is believed that the expression of various isoforms plays a role in both myometrium quiescence and transition to a contractile phenotype during labor [66]. Alternative splicing of CRH-R mRNA results in different receptor isoforms (such as CRH-R1α and CRH-R1d) with different activities that generate a more contractile phenotype [67]. In myometrial strips from laboring and nonlaboring pregnant women, it was shown that CRH could inhibit spontaneous contractility of nonlaboring but not of laboring myometrium, acting via the CRH-R1 receptor. This effect further implies that CRH may exhibit a dual role at the myometrium [68].

It is suggested that CRH-R1 promotes relaxation of smooth muscles via intracellular signals that inhibit phosphorylation of the contractile protein myosin light chain (MLC20). The decreased expression of proteins such as Gα_s-protein (a G protein subunit that activates the cAMP-dependent pathway by activating adenylate cyclase) towards the end of pregnancy turns the balance towards myometrial contractility. The increased expression of CRH-R2 protein toward onset of labor increases MLC20 phosphorylation and myometrial contractility [67].

CRH-R1 and CRH-R2 have been identified both in the upper and lower uterine segment of laboring and nonlaboring human myometrium, but during labor CRH-R1 decreases significantly at the upper segment but not at the lower one. This decreased expression may be related to the fact that during labor the fundus of the uterus switches to a highly contractile state, while the lower segment remains relatively quiescent [69]. Recently a new isoform of CRH-R has been identified in human pregnant but not in non-pregnant myometrium. This isoform is termed CRH-R1β/d and shows similarities with both CRH-R1β and -R1d, such as reduced affinity for CRH, negligible signaling, and mainly cytoplasmic localization. Its expression is maximum at the early third trimester, and it is increased by estradiol-17β [70].

3.3. The Effect of Urocortins and Interleukins on CRH Receptors. CRH receptors may also be activated by other agonists such as the urocortins. Urocortin 2 (UCN 2) is a CRH-R agonist that is expressed in human pregnant myometrium and interacts with CRH-R2 receptors and through a signal transduction pathway involving the protein kinase C (PKC), the extracellular signal-regulated kinases ERK1/2, and the RhoA/ROK. Urocortin 2 interaction stimulates MLC20 phosphorylation, which in turn induces myometrial contractility [71]. Although urocortins 2 and 3 are expressed

in the human placenta, it is not known if they act by increasing myometrial contractility during labor [72]. Recent data have reported that, in human placentas from women with PPROM and chorioamnionitis, inflammatory events are associated with changes to the CRH-related mechanism of labor, such as increased CRH, UCN 2, and CRH-R1 mRNA expression and decreased UCN 3 and CRH-R2 expression [73].

Immune system responses are also associated with changes to the CRH-related mechanisms of labor. Experiments on both term and preterm, laboring and nonlaboring human myometrium show that active labor is associated with increase of CRH-R1α and CRH-R1β mRNA in term and preterm myometrium. IL-1β induces CRH-R1$_T\alpha$ mRNA and prostaglandin synthetase 2 (PTGS2) mRNA and protein levels, decreases CRH-R1β mRNA, and impairs CRH-induced cAMP production. It may therefore be hypothesized that IL-1β acts as a regulator of CRH-R1 expression and function that contributes to a preparatory contractile environment necessary for the initiation of labor [74].

CRH may also act on both term and preterm cervical fibroblasts and increase IL-8 expression, which is an important mediator of cervical ripening [75].

3.4. Clinical Applications of CRH in Human Labor. Several blood, amniotic fluid, or vaginal fluid markers have been proposed as predictors of preterm labor, including interleukins, cortisol, fibronectin, ADAM-8, ITAC, estriol, a-FP, b-HCG, and others [76–81]. Many of these studies have evaluated the clinical usefulness of CRH measurements to predict preterm labor [56, 82, 83]. Urocortin may also serve as a biomarker of labor in women with threatened preterm birth before 34 weeks [84]. However, these observations have mostly diagnostic and not therapeutic benefit, since no CRH/CRH receptor antagonist has so far been identified that can be used in humans to prevent preterm labor.

4. Interactions between Progesterone and CRH in Labor

In human placental cells progesterone inhibits CRH production, but this effect is modulated differently from PR-A and PR-B, since PR-A overexpression leads to decreased CRH promoter activity, while PR-B increases CRH promoter activity in the presence of exogenous progesterone [85]. Progesterone induces CRH-R1 and CRH-R1β variants, which lead to increased action of CRH, effecting under these conditions uterine quiescence during pregnancy. The effect of functional progesterone withdrawal on these receptors may enable myometrial contractions during labor [70]. CRH causes relaxation of term and preterm human myometria, and progesterone acts synergistically to enhance it in term but not in preterm myometrium. This provides extra evidence that progesterone withdrawal in late pregnancy may lessen the relaxant effect of CRH [86].

CRH may also itself contribute to progesterone withdrawal towards labor. Experiments in human placental trophoblasts show that CRH decreases placental production of progesterone by reducing the levels of the progesterone synthesis enzymes cytochrome 450 CYP11A1 and HSD3B1. In contrast, treatment with a CRH antagonist increases progesterone production. This effect of CRH on progesterone production is mediated through a PKC-dependent pathway [87].

5. Conclusion

Progesterone is a highly important hormone that contributes to the maintenance of pregnancy. Progesterone withdrawal occurs either by decreasing plasma levels in animals or by functional withdrawal mechanisms in humans. CRH is another significant hormone which, like progesterone, acts via different mechanisms either to support pregnancy or to facilitate the process of labor. These two hormones interact with each other to promote myometrial quiescence or contractility. Inflammatory immune responses such as infiltration of uterus with immune cells and production of cytokines contribute significantly to the mechanisms of labor. Progesterone and CRH regulate some of these immune actions. Despite intensive research for many years, there are still a large number of enigmas and areas of controversy regarding human labor and the role of both hormones and inflammation. Animal models do not show similar mechanisms to those of the human fetus and this is one of the obstacles to attaining better understanding of human labor. In the future, clinical applications based on the actions of progesterone, CRH and inflammatory immune responses may be essential for the prevention of preterm labor, which is today a major cause of neonatal morbidity and mortality.

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Review Article

Current Concepts of Immunology and Diagnosis in Amniotic Fluid Embolism

Michael D. Benson

Department of Obstetrics and Gynecology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

Correspondence should be addressed to Michael D. Benson, m-benson@northwestern.edu

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Amniotic fluid embolism (AFE) is one of the leading causes of maternal mortality and morbidity in developed countries. Current thinking about pathophysiology has shifted away from embolism toward a maternal immune response to the fetus. Two immunologic mechanisms have been studied to date. Anaphylaxis appears to be doubtful while the available evidence supports a role for complement activation. With the mechanism remaining to be elucidated, AFE remains a clinical diagnosis. It is diagnosed based on one or more of four key signs/symptoms: cardiovascular collapse, respiratory distress, coagulopathy, and/or coma/seizures. The only laboratory test that reliably supports the diagnosis is the finding of fetal material in the maternal pulmonary circulation at autopsy. Perhaps the most compelling mystery surrounding AFE is not why one in 20,000 parturients are afflicted, but rather how the vast majority of women can tolerate the foreign antigenic presence of their fetus both within their uterus and circulation?

1. Introduction

When first described in the first half of the 20th century, amniotic fluid embolism was presumed to be the result of the physical obstruction of the maternal pulmonary circulation by fetal material contained within amniotic fluid [1, 2]. In the initial cases described at autopsy, abundant fetal material was seen in pulmonary vessels. The disease is rare, with an incidence ranging from one in 600 to one in 80,000, perhaps because there is no established laboratory marker diagnostic suitable for both survivors and fatalities alike [3, 4]. AFE is one of the leading causes of maternal mortality in the United States and causes roughly 10% of all maternal deaths in developed countries [5–7]. Perhaps an equal concern is the significant maternal morbidity that results in survivors. In 48 British survivors, four had neurological injury, two had thrombotic events, one had renal failure, and another had septicemia [8]. 94% were admitted to the ICU. In an Australian study, three of thirteen survivors suffered a cerebral infarction [9]. The morbidity and mortality for the fetus is also significant. In the British registry, among the fifteen women who died of AFE, eleven of their babies died also, and one of the survivors had hypoxic ischemic encephalopathy [7]. Among the thirty-one surviving women with known newborn outcomes, nine newborns died or suffered serious injury. Although a rare complication of pregnancy, the high rates of injury for both mother and newborn provide compelling arguments for a better understanding of the mechanism of disease.

While "embolism" is contained with the disease name both in its original Spanish and English descriptions, there are a number of confounding clinical observations about AFE that cast doubt on this mechanism of disease [1, 2]. First, mechanical obstruction of pulmonary blood flow is not reliably seen in AFE [10]. Second, clinical events, common in AFE, coagulopathy, adult respiratory distress syndrome, and neurological symptoms, are not typical in pulmonary embolism. Perhaps as a result, observers began speculating about a possible role for an immune mechanism as early as 1950 [11]. Yet with the disease appearing rarely, suddenly, and unpredictably, obtaining relevant evidence in humans has proven unusually difficult. Unfortunately, animal studies have provided little insight other than to suggest there is little to learn from animal models. Illness has been induced in animals (rabbits and dogs) with the intravascular injection of heterologous (human) amniotic fluid contaminated by

meconium [2]. In particular, autologous amniotic fluid introduced into the maternal circulation in monkeys seems to be entirely benign [12, 13].

2. Anaphylaxis

The first specific suggestion of anaphylaxis as a mechanism of AFE was made by Attwood in 1956 [14]. Benson and Lindburgh suggested that this hypothesis was testable in humans by testing women acutely ill with AFE for serum tryptase [15]. Tryptase, a serine protease with a half-life of several hours, is released by mast cells along with histamine when they degranulate in response to IgE crosslinking on the cell surface in the presence of antigen. Although the specific function of tryptase in anaphylaxis is unknown, with a half life measured in hours instead of the minutes of histamine, the protein has proven useful in the diagnosis of anaphylaxis. Urinary histamine has also been used to diagnose anaphylaxis as a small percentage of histamine is excreted into the urine, unmetabolized [16].

In considering the role of mast cell degranulation in the mechanism of amniotic fluid embolism, the serological evidence should be considered separately from the histological data since, in both cases, the evidence is somewhat mixed. Serum tryptase testing in AFE points against a role for AFE while tryptase staining in pulmonary histology at autopsy provides a more nuanced picture.

In 2001, a study of nine women with amniotic fluid embolism was published in which specific evidence of anaphylaxis was sought [17]. Seven of the nine patients had sera stored as part of the Japanese National Maternal Mortality Surveillance project while two patients were American survivors enrolled in a protocol specifically created to collect acute samples in AFE patients. Five patients were negative for serum tryptase, one was negative for urinary histamine, and one was negative for both. There were no positive findings for either serum tryptase or urinary histamine in any of the patients.

Since this study, elevated serum tryptase levels have been reported in fatal cases of amniotic fluid embolism. In three such reports, serum tryptase levels have been only slightly elevated, raising serious questions about the premise that anaphylaxis reflects the underlying mechanism [18–20]. In the fourth case of AFE, the authors also looked at postmortem serum tryptase levels in nonpregnant, non-AFE cases such as pulmonary embolism and traffic accidents [21]. While the serum tryptase level in the AFE case was six times the upper level of normal, 17 hours after death, the one confirmed anaphylaxis case had a tryptase levels over 60 times normal, 24 hours after death. Finally tryptase levels in a fifth, surviving case of AFE were normal [22]. The slight serum elevations in fatal cases of AFE are below that normally seen for postmortem values seen in anaphylaxis and do not support the hypothesis that mast cell degranulation plays a central role in the pathophysiology of amniotic fluid embolism.

With 100% of 6 AFE patients testing negative for tryptase in the largest series to date, the best that can be said about serum tryptase in AFE is that it has very poor sensitivity [17]. However, there are objections to the specificity of the test as well. Unless tryptase is measured in both normal parturients and mortally ill obstetric patients with illnesses other than AFE, the specificity of tryptase will remain unknown. It is possible that tryptase is elevated in specific obstetric populations without AFE. At the present, serum tryptase in AFE can only be considered an investigational tool with both normal and elevated levels neither confirming nor excluding the diagnosis.

The evidence from maternal pulmonary histology is more complex concerning the role of anaphylaxis in AFE. Two studies by Italian pathologists did find evidence of mast cell degranulation in the maternal pulmonary vasculature. The first study performed mast cell counts per fixed area by using an immunohistochemical stain for tryptase [23]. The investigators compared the six fatal AFE cases with six anaphylactic deaths, five traumatic pregnancies deaths, and six traumatic deaths in nonpregnant women. Remarkably, the AFE group had higher mast cell densities than the anaphylactic group and much higher than the other two control groups. In the second study using a similar approach, the investigators were able to show a large increase in extracellular tryptase consistent with mast cell degranulation in the eight women in the AFE group compared to the six pregnant women who succumbed from traumatic injury [20]. One case report of AFE failed to find immunohistochemical evidence of mast cell degranulation at autopsy, but the patient in question died minutes after a head on motor vehicle accident and did not clearly have AFE [24].

In summary, the available evidence suggests that mast cell degranulation does take place in the lungs in fatal AFE cases and does not do so in other mortal pregnancy conditions. This is hard to reconcile with the general lack of significant circulating tryptase elevations in AFE. These observations together suggest that pulmonary mast cell involvement in fatal cases may be a secondary process and not necessarily the primary mechanism of AFE. This possibility is all the more intriguing because mast cell degranulation can occur as a result of complement activation [25]. In considering the mechanism of disease, evidence of tryptase release cannot be taken as proof as a primary role for anaphylaxis without the simultaneous measurement of complement and confirming that activation did not take place.

Finally before leaving the subject of anaphylaxis and amniotic fluid embolism, the suggestion that the disease be renamed "Anaphylactoid Syndrome of Pregnancy" should be considered [26, 27]. While the term "anaphylaxis" can refer loosely to the clinical symptoms resulting from any process resulting in mast cell degranulation, it is more commonly used to denote a process mediated by IgE binding to antigen [28]. In contrast, the common usage of "anaphylactoid" is used specifically to refer to nonimmune-mediated degranulation of mast cells—not involving IgE [29]. The best known example in humans of an "anaphylactoid" reaction is the histamine release that is sometimes seen in people with their first exposure to intravenous X-ray contrast [30]. Potentially as fatal as IgE-mediated anaphylaxis, the presumption underlying anaphylactoid reactions is that they do not result from

a remembered antigenic response since there has not been previous exposure. Beyond the fact that mast cell degranulation is by no means a proven mechanism of AFE is the idea that all speculation regarding disease mechanism centers on some type of maternal immune response to fetal antigen [31]. No mechanism has been proposed for AFE in which mast cells degranulate in the absence of antibody-antigen interaction. Although "Anaphylactoid Syndrome of Pregnancy" has not gained widespread acceptance, available evidence does not support changing the name of AFE, and the term should be abandoned.

3. Complement Activation in AFE

The frequency of adult respiratory distress syndrome as a sequela of AFE suggested to Jacob and Hammershmidt in 1982 [32], and Hammershmidt et al. [33] that complement activation may have a role to play in the pathophysiology of disease. The first series in which serum complement levels were evaluated in AFE patients found that both C3 and C4 were significantly depressed in all eight AFE patients in which measurements could be done [17]. A control group of twenty-three normal laboring patients all had complement levels in the normal range. Furthermore, in a case report concerning possible "mild" AFE in which a patient had transient shortness of breath, palpitations, and laboratory evidence for a coagulopathy, complement levels were also depressed [34]. However, in a separate series of AFE patients in Japan, the average C3 level was low normal (71 mg/dL) while the average C4 levels were depressed (13.9 mg/dL) [7]. There were no differences in the means between those who survived and those who died. The Italian group that did histology studies for tryptase also considered complement activation in their most recent paper [20]. They found depressed amounts of C3a in the pulmonary circulation of the eight AFE patients compared to the six pregnant women dying of trauma. The investigators suggested that the diminished pulmonary C3a was consistent with complement activation. As noted previously, there was evidence for tryptase release in the same histology samples. It is possible, if not likely, that complement activation may have been the initial immune response which then resulted in a secondary degranulation of mast cells.

With both serologic and histologic evidence, the complement pathway appears more promising than anaphylaxis as a possible mechanism of disease. However, as a diagnostic tool, both sensitivity and specificity are poorly characterized. As with tryptase, complement levels in AFE remain an investigational tool and should not be used to either confirm or refute a clinical diagnosis.

During the investigations into complement activation in AFE, an interesting footnote should be mentioned. It appears that some degree of complement activation during normal labor is physiologic and peaks at the moment of birth. Two separate studies in which serial complement levels were obtained in normal laboring parturients have been performed. The first was done as part of the 2001 study looking at complement and tryptase levels in AFE [17]. A healthy control group of thirteen American women and nine Japanese women had complement levels obtained on admission to the hospital in labor and on the first postpartum day. A within pairs analysis was performed, and both C3 and C4 demonstrated statistically significant declines, 8% and 5%, respectively. In a second study of twelve healthy American women, serial complement levels were obtained several times during labor and within an hour postpartum [35]. Again, the decreases in complement levels were highly statistically significant with C3 dropping an average of 15% and C4 diminishing by 11%. In both studies, all complement levels remained within the normal range. Taken together, they suggest that complement activation peaks at or shortly before birth and that levels start to return to normal in the postpartum period [10]. The biological significance of complement activation during normal parturition is unknown but is consistent with current evidence that the initiation of labor may be mediated by an inflammatory response rather than simply a fluctuation in hormones [36].

4. Clinical Diagnosis of AFE

There is a reasonably broad consensus in the published literature on the clinical diagnosis of AFE without the reliance on laboratory markers. In general, the definitions of the disease point to one or more of the following clinical signs first characterized by Courtney in 1974: cardiovascular instability, respiratory distress, coagulopathy, or coma/seizures in the absence of other explanations [37]. Benson used the same definition for his research protocols but stipulated a forty-eight hour time limit from birth [10]. The Japan Consensus Criteria for the diagnosis of AFE was similar with the omission of neurological symptoms and a twelve-hour limit from birth [7]. Conde-Agudelo and Romero of the National Institutes of Health adopted the Benson definition for clinical diagnosis in their 2009 review article, which was also used in an Australian population-based cohort study [9, 38]. In sum, the clinical definition of AFE has remained reasonably constant over the past four decades.

The UK Obstetric Surveillance System criteria for AFE are a bit more nuanced although still clearly similar to the Japanese and the American clinical definitions [39]. The British do not define a time limit from parturition and include maternal hemorrhage, in general, as well as coagulopathy. Unlike the other clinical definitions, they do consider acute fetal compromise in the absence of other clear cause as a diagnostic sign of AFE. They also include nonspecific premonitory symptoms such as restlessness or numbness, agitation, and tingling. Perhaps most significantly, the British include one laboratory test as being sufficient for the diagnosis regardless of clinical course—the finding of fetal material in the maternal pulmonary vasculature at autopsy.

There are several nuances to the clinical diagnosis of AFE. All definitions assign the diagnosis with the proviso that alternative diagnoses have been excluded although this does not preclude other comorbidities such as abruption. Yet several other clinical findings are associated with AFE even if they are not always considered diagnostic in their own right.

For instance, uterine atony has been closely associated with AFE in several reports [37, 40]. Beyond being a diagnostic criteria in the United Kingdom, several case reports have also described fetal bradycardia occurring early in the onset of amniotic fluid embolism [6, 41, 42]. Regarding coagulopathy and the diagnosis of amniotic fluid embolism, at least six case reports have described coagulopathy alone as the sole clinical sign/symptom of AFE [43–48]. It should also be noted that all clinical definitions of the disease described above anticipate that coagulopathy occurring in isolation may be the sole clinical sign/symptom of AFE [7, 10, 37–39].

5. Laboratory Diagnosis of AFE

An examination of the current state of laboratory diagnosis of AFE is fraught with conflicting and paradoxical evidence. To be sure, AFE as a clinical entity emerged from the autopsy room with eight maternal deaths at the University of Chicago defining the first series of the disease. These first cases had large amounts of apparent fetal material in the maternal pulmonary vasculature. It is, therefore, no surprise that finding any fetal material in the maternal circulation has been presumed to confirm a diagnosis of AFE. However, this is clearly not the case as there have been any number of both individual case reports and case series in which fetal material is present in the maternal circulation in women not experiencing AFE. To add confusion to the issue, the limited autopsy studies available draw quite a different picture from studies in living patients.

There are a growing number of reports in the literature in which fetal material is found in the maternal circulation of living parturients who do not have amniotic fluid embolism. Perhaps the earliest reports are those of two separate cases in which fetal material was found in the maternal circulation of the uterus at the time of peripartum hysterectomy in women who did not have AFE [49, 50]. In the 1980s, at least three studies were done in which pulmonary artery samples were taken from critically ill pregnant patients, looking for fetal material [51–53]. Any number of these aspirates had fetal material, ranging from squamous to mucin to lanugo in women who did not have AFE. This is further supported by a recent pathology study of 57 peripartum hysterectomy specimens for women who were experiencing excessive bleeding [54]. There was no relationship between the presence or absence of uterine intravascular fetal material and the etiology of maternal hemorrhage. Seven diagnoses were attributed to the etiology of the hemorrhage: uterine rupture, abruption, uterine atony, placenta previa, accreta, coagulopathy, and retained placenta. Specifically, women in all seven diagnostic categories had both the presence and the absence of fetal material including those with disseminated intravascular coagulopathy, one of the signs of amniotic fluid embolism.

In contrast, two case reports relied on the observation of intravascular fetal material in the uterus to confirm a diagnosis of amniotic fluid embolism. In the first, the authors assume that they "prevented AFE" by ligating the uterine vessels with the fetal material in it [55]. They saw vernix and air bubbles entering the uterine vein and ligated the uterine vessels without removing the uterus. The patient's only morbidity was a subclinical laboratory coagulopathy. In the second case report, the authors similarly assume that a fatal case was averted because the fetal material did not enter the pulmonary circulation [43]. They observed fetal material within the uterine veins after a peripartum hysterectomy on histology studies. In light of the numerous reports cited previously, the currently available evidence does not support using the presence or absence of intravascular fetal materials in living patients to confirm or refute a diagnosis of AFE.

To make matters more confusing, the limited data from autopsy studies suggest that intravascular fetal material is both sensitive and specific for AFE. In 1998, Japanese investigators used a immunohistochemical stain for a fetal antigen, STN, to make a diagnosis of AFE at autopsy [56]. All four AFE patients had positive staining of the pulmonary vasculature while the control group of four women who died from non-AFE causes did not. A second study of twenty-seven maternal deaths found fetal material in 100% of AFE patients and none in those dying of non-AFE causes [57]. It should be noted that the study findings were problematic since the authors began with the assumption that any fetal material in the maternal vasculature was diagnostic of AFE. With that said, they did not find fetal material in any cases that were obviously not AFE such as traumatic injury. The previously cited Italian studies also found fetal material in the pulmonary vasculature in AFE patients at autopsy while not finding it in pregnant women dying of other causes [20, 23]. The evidence from several dozen autopsies on women with and without AFE suggests that the finding of fetal material in the maternal circulation at autopsy is specific for AFE. This fact is utilized in the diagnostic criteria of the United Kingdom as the only laboratory test considered diagnostic [39]. Similarly, histology at autopsy seems to be sensitive for AFE although, in all studies, specialized stains were used to look for fetal material.

The difference in the presence of fetal material in the maternal vasculature between those dying and surviving is seen in another study of 135 women with AFE in Japan [7]. In this study, serum levels of a specific fetal antigen, STN, were higher in those succumbing to the disease. However, the levels between survivors and those dying did overlap sufficiently to prevent STN levels alone from being a very useful, discriminating diagnostic marker for AFE.

As with tryptase and complement, the presence or the absence of fetal material in the maternal circulation of living women cannot either confirm or refute the diagnosis of AFE. However, the limited available evidence suggests a somewhat different conclusion at autopsy, where the presence of intravascular fetal material does seem to be specific for AFE. It is unclear why there should be a difference in the sensitivity and specificity of intravascular fetal material between the living and the dead. An obvious explanation is that a greater amount of fetal material may be more mortal and thus more detectable at autopsy. Yet this does not seem entirely satisfactory on further examination since the amount of fetal material in fatal AFE cases was not necessarily massive and indeed present only in microscopic amounts.

6. Conclusions

Much has been published about amniotic fluid embolism that, in light of current evidence, needs to be revised. The evidence does not support renaming the disease "Anaphylactoid Syndrome of Pregnancy." The only laboratory test that is diagnostic at this time is the finding of fetal material in the maternal pulmonary circulation at autopsy. Serum tryptase levels should not be used to affirm or refute a diagnosis of AFE. Similarly, while there is better evidence concerning complement, it too remains an investigational tool and is not yet a reliable diagnostic marker. There is enough of a worldwide consensus on diagnostic criteria to permit reasonably consistent clinical diagnoses in the absence of confirmatory laboratory testing at the present. Generally, the diagnosis relies on one or more of four signs or symptoms occurring during pregnancy or shortly thereafter: cardiovascular collapse, respiratory distress, coagulopathy, and coma or seizures. Finally, intravascular fetal material in living women clearly does not necessarily result in AFE although it may not be either benign or physiologic. Perhaps the best way forward is the continued use of a difficult but achievable methodology—looking at immune markers in sera obtained from healthy controls and acutely ill pregnant women, both with and without AFE. The study of AFE is important both because it causes significant morbidity and mortality among mothers and their babies and also because a better understanding of this disease may lead to a more insight into immune tolerance in general. The overarching importance of the question underlying the mechanism of AFE is not so much why does a specific patient get the disease but rather how can women tolerate the presence of so much foreign antigen within both their uterus and their circulation?

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Review Article

IgG Placental Transfer in Healthy and Pathological Pregnancies

Patricia Palmeira,[1,2] Camila Quinello,[2] Ana Lúcia Silveira-Lessa,[3] Cláudia Augusta Zago,[2] and Magda Carneiro-Sampaio[1,2]

[1] *Departamento de Pediatria, Faculdade de Medicina, Universidade de São Paulo, Av. Dr. Enéas Carvalho de Aguiar, 647, Cerqueira César, São Paulo, SP 05403-900, Brazil*
[2] *Laboratório de Investigação Médica (LIM-36), Instituto da Criança, Hospital das Clínicas, Av. Dr. Enéas Carvalho de Aguiar, 647, Cerqueira César, São Paulo, SP 05403-900, Brazil*
[3] *Departamento de Parasitologia, Instituto de Ciências Biomédicas, Universidade de São Paulo, Av. Prof. Lineu Prestes, 1374, Cidade Universitária, São Paulo, SP 05508-000, Brazil*

Correspondence should be addressed to Patricia Palmeira, patpalm@usp.br

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Placental transfer of maternal IgG antibodies to the fetus is an important mechanism that provides protection to the infant while his/her humoral response is inefficient. IgG is the only antibody class that significantly crosses the human placenta. This crossing is mediated by FcRn expressed on syncytiotrophoblast cells. There is evidence that IgG transfer depends on the following: (i) maternal levels of total IgG and specific antibodies, (ii) gestational age, (iii) placental integrity, (iv) IgG subclass, and (v) nature of antigen, being more intense for thymus-dependent ones. These features represent the basis for maternal immunization strategies aimed at protecting newborns against neonatal and infantile infectious diseases. In some situations, such as mothers with primary immunodeficiencies, exogenous IgG acquired by intravenous immunoglobulin therapy crosses the placenta in similar patterns to endogenous immunoglobulins and may also protect the offspring from infections in early life. Inversely, harmful autoantibodies may cross the placenta and cause transitory autoimmune disease in the neonate.

1. Introduction

Anti-infectious fetal protection is provided by several factors acting together. The uterine cavity contains innate immune detection and effector systems that maintain sterility, detect infection and, under conditions of substantial microbial invasion, induce expression of mediators that could accelerate lung maturation and induce a preterm labor to deliver the fetus from a threatening environment [1]. The vaginal tract, which is normally colonized with multiple microorganisms, is separated from the normally sterile intrauterine compartment by the cervical plug, which contains several antimicrobial proteins and peptides (APPs), including lactoferrin and α-defensins. Inside the uterine cavity, the amniotic fluid contains acute phase proteins, such as soluble CD14 and lipopolysaccharide- (LPS-) binding protein (LBP), which modulates the endotoxic activity of LPS and cationic membrane-active APPs, such as lactoferrin, bactericidal/permeability-increasing protein, histones, and defensins [2]. In preterm labor, increased concentrations of group II phospholipase A2 are found, and this enzyme has been associated with a remarkable potency against Gram-positive bacteria [3, 4].

At birth, the neonate presents an increased susceptibility to infectious agents due to functional immaturity of his/her immune system. Some functions are particularly immature, whereas other aspects are functional at birth even in extremely preterm newborn infants. Neutrophils have a small storage pool at birth, and this cell lineage is less responsive to chemoattractants than later in development. Monocytes/macrophages are reported to be functionally adequate but have limitations in chemotactic responsiveness. Infant blood monocytes produce less IFN-α, IFN-γ, and IL-12 subunit p70 (IL-12 p70) than cells obtained from adults. However, production of these cytokines rapidly increases between birth and 1 or 2 years of age. In contrast, infant cells

show a greater capacity to produce IL-10 and to induce IL-17-producing helper T cells (Th17 cells) in response to Toll-like receptor (TLR) stimulation by producing IL-6 and IL-23 [2]. Furthermore, individual infant cells are less able than adult cells to produce multiple cytokines simultaneously in response to TLR agonists; that is, infant cells are less polyfunctional [5]. The predominance of a Th17-like pattern combined with considerable IL-10 production may contribute to diminished T helper type 1 (Th1) responses, resulting in greater susceptibility to intracellular infections and diminished vaccine responses during infancy [6].

Neonatal T $CD4^+$ cells present an intrinsic immaturity with a diminished capacity to generate memory cells and reduced Th1 effector functions such as the production of less IFN-γ and lower CD40L expression. These deficiencies seem mainly to be related to the fact that the cells are still naive, having met few antigens [7]. Thymic recent emigrants (TRECs), which are T cells recently migrated from the thymus, are present in a large proportion in the periphery of human infants, and these TRECs are impaired in their acquisition of Th1 function [8]. $CD4^+$ T cell responses, but not $CD8^+$ T cell responses, develop more slowly in infants than in adults after primary infection with cytomegalovirus or herpes simplex virus [9]. In addition, responses to some vaccines, such as vaccines for hepatitis B virus and oral poliovirus vaccine, result in less Th1 activity and a bias toward Th2 function [10]. The ability of proinflammatory cytokines to induce spontaneous abortion is likely to be an important reason for the strong bias of the maternal and fetal immune systems of multiple mammalian species towards Th2-cell-polarizing cytokines [1, 11]. The Th2 locus is hypomethylated in both human and mouse infants, contributing to the expression of these cytokine genes, which corresponds to the propensity for Th2-polarizing cytokine responses in infants [12, 13]. Thus, infants have a dominant anti-inflammatory cytokine profile that seems to be induced during fetal life [7]. It has been demonstrated that in the in utero environment, $CD4^+CD25^{hi}Foxp3^+$ regulatory T cells dominate the fetal circulation, suppressing reactivity to non-inherited maternal antigens [14] and possibly promoting a generally suppressive environment.

Regarding neonatal antibody responses, several studies have shown a delayed onset, lower peak levels, a shorter duration, differences in the distribution of IgG isotypes (with infants showing lower IgG2 than adults), and lower affinity and reduced heterogeneity. Antibody responses to thymus-independent type 2 antigens (including bacterial polysaccharides) are also deficient [15]. There is no transplacental transfer of complement system elements, and neonates have relatively low levels of some components [16]. Furthermore, neonatal and infantile B cells have low expression of CD21 (complement receptor 2), which explains the inadequate response to polysaccharides [17]. Interestingly, the increase in CD21 levels that occurs during development coincides with the response to polysaccharides [18].

Considering that after exposure to each new microbe it takes time to develop each specific protective immune response, the placental transfer of maternal immunoglobulins to the fetus is a specific adaptative mechanism that, to some extent, minimizes the deficiencies in antibody production and confers short-term passive immunity. Moreover, additional immune response support is given by the mother through breast milk, which contains functional nutrients and IgA antibodies that provide efficient protection directly after birth by preventing adherence of infectious agents on the mucosal membranes and ultimately their entrance into tissues.

2. IgG Placental Transfer Is Mediated by FcRn

In humans, substances that pass from maternal blood to fetal blood must traverse the histological barrier, which consists of two cell layers: the multinucleated syncytiotrophoblasts (STBs) and endothelial cells of the fetal capillaries. Furthermore, fibroblasts and Hofbauer cells (i.e., placental macrophages) are found in the villous stroma and are presumably involved in the binding and trapping of immune complexes [19].

Although this barrier separates the blood in maternal and fetal circulation, it is not a simple physical barrier. A wide range of substances, including nutrients and solutes, are efficiently transferred actively or passively through the placenta to the fetus, and this mechanism is essential for normal fetal growth and development. Most low molecular mass compounds (<500 Da) simply diffuse through the placental tissue interposed between the maternal and fetal circulation. Some low molecular weight substances, such as ions and amino acids, show unidirectional transfer across the placenta. Substances of very high molecular weight do not usually traverse the placenta, but there are a few exceptions, such as immunoglobulin G (IgG), which has a molecular mass of approximately 160 kDa.

Of the five antibody classes, only significant amounts of IgG are transferred across the placenta. On the basis of the observation that whole IgG molecules or Fc fragments of IgG pass into the fetal circulation more readily than $F(ab')^2$ fragments [20], it was hypothesized that IgG Fc receptors (FcγRs) on placental cells may be involved in IgG transfer across placenta. Later, it was established that this specific transport of IgG is carried out by the neonatal Fc receptor (FcRn) [21, 22]. This has been demonstrated unequivocally in *ex vivo* perfused placenta by comparing the transport of a recombinant, humanized IgG1 antibody with that of a mutated variant that does not bind to FcRn [23]. FcRn is composed of an integral membrane glycoprotein with an apparent molecular weight of 40–45 kDa for the α-chain, which is noncovalently associated with β2-microglobulin (β2 m) [24]. Thus, while the major ligands of FcRn are IgG and albumin, FcRn is most closely structurally related to major histocompatibility complex (MHC) class I molecules, with which it shares 22%–29% sequence homology. In contrast to other Fcγ-receptors, FcRn exhibits a characteristic pH-dependency of IgG binding, demonstrating a high affinity for IgG at pH 6.0, but 100-times lower affinity at physiological pH (7.4) [25]. Thus, FcRn is unable to bind IgG at the apical side of STB facing the maternal blood.

It is, therefore, assumed that IgG present in high concentrations in the maternal circulation (10–20 mg/mL) is taken

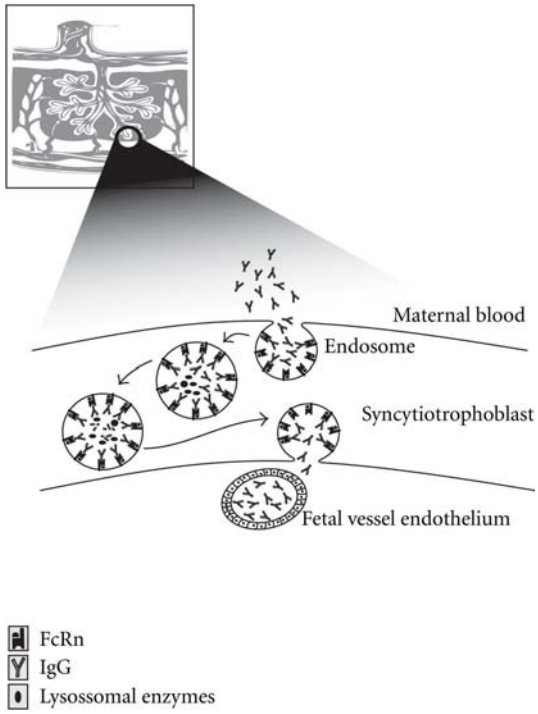

Figure 1: IgG transfer from the mother to the fetus occurs during pregnancy across the syncytiotrophoblasts of the placenta. Syncytiotrophoblasts are bathed in maternal blood and internalize maternal IgG in endosomes. FcRn is expressed on the internal surfaces of the endosome. Upon acidification in the endosome, maternal IgG bound to FcRn is protected from degradation by lysosomal enzymes and then is transcytosed. The endosomes fuse with the membrane on the fetal side of the syncytiotrophoblast, where the physiological pH promotes the dissociation of IgG from FcRn to the fetal circulation. High levels of IgG antibodies cause IgG degradation due to the saturation of FcRn receptors.

up by fluid-phase endocytosis by STB and then binds to the FcRn in the acidic environment of endosomes [26]. Bound IgG may then be transcytosed to the basolateral side, where it is released upon exposure to neutral pH (7.4). The FcRn molecule may then be recycled to the maternal membrane to perform additional rounds of transcytosis, as observed in other systems [27]. Therefore, pH-dependent binding of IgG to FcRn allows for IgG transport through a cell layer and down a concentration gradient of IgG [28–30] (Figure 1).

Transcytosed IgG may or may not pass through the stroma before reaching the fetal blood vessels. It remains controversial as to whether FcRn is also expressed in the fetal vessel endothelium, where greater evidence exists for the action of alternative Fc receptors in further movement of IgG [31, 32]. This IgG transport model is supported by the *in situ* localization of IgG and FcRn subunits and by studies investigating IgG transport in *ex vivo* perfused placentae [33]. The FcRn α-chain has been found to be localized mainly in intracellular vesicles and to a minor extent at the apical membrane of STB of first trimester and term placentae [26, 33, 34].

The function of FcRn also extends to many other sites within the body, where it plays an important role in modulating lifelong humoral and cell-mediated immune responses. It is also expressed in both endothelial and bone marrow-derived cells and plays an integral role in protecting IgG from catabolism, which allows IgG to be recycled to the cell surface and back into the bloodstream, extending its half-life in the serum of adults [35]. FcRn is also expressed in many other tissues in the adult animal, including barrier sites such as the blood-brain interface, the glomerular filter in the kidneys and the intestinal epithelium, where its function of modulating IgG transport to promote host defense or to control immune-complex deposition is still speculative [36].

To be transferred through human placenta, maternal IgG must cross the STBs, the stroma of the intravillous space, and the fetal vessel endothelium. These tissues express unique patterns of various types of Fc receptors of IgG including FcγRI, FcγRII, and FcγRIII. In the placenta, FcγRI has been found in the loose connective tissue, mononuclear phagocytes, and the Hofbauer cells, which are morphologically defined as macrophages due to their ability to perform phagocytosis and to interact with IgG. Trophoblast cells in term placentae express both FcγRIII and FcRn. Placental FcγRIII is a membrane-spanning FcγRIIIa isoform, which is predominantly expressed by Natural Killer (NK) cells. The binding of FcγRIII (also called CD16) on NK cells to immune complexes or IgG on target cells, or treatment with an anti-CD16 monoclonal antibody to crosslink membrane spanning FcγRIII induces NK cell activation. This activation leads to upregulation of the transcripts for cytokines such as IFN-γ and TNF-α [37, 38]. These observations indicate that FcγRIIIa on trophoblasts may bind immune complexes or antibody-coated particles in the maternal circulation and may induce the transcription of cytokines or trigger cell-mediated immunity.

Fetal endothelial cells in placenta express FcγRII and FcRn although data regarding FcRn expression in the endothelium are still conflicting [39].

3. Placental Transport of IgG Depends on Maternal Levels

The newborn IgG antibodies' levels usually correlate with maternal ones (Figure 2); however, the IgG binding to FcRn receptor can be saturated. Thus, the amount of IgG transmitted depends on the amount of cell surface receptors, because unbound IgG molecules are digested by lysosomal enzymes inside the vesicles [40]. This has been reported in several works performed in certain regions of Africa showing lower cord/maternal placental transfer ratios of total IgG, indicating that this limitation of active placental transfer of antibodies is related to the higher maternal IgG levels common in Africa [41–43]. It was reported by Michaux et al. [44] that total IgG concentrations in cord sera tend to be lower than in their mothers when total IgG levels in maternal serum reached 15 g/L. This is in agreement with other works that have demonstrated significant negative correlations between maternal levels of IgG and placental transfer ratios

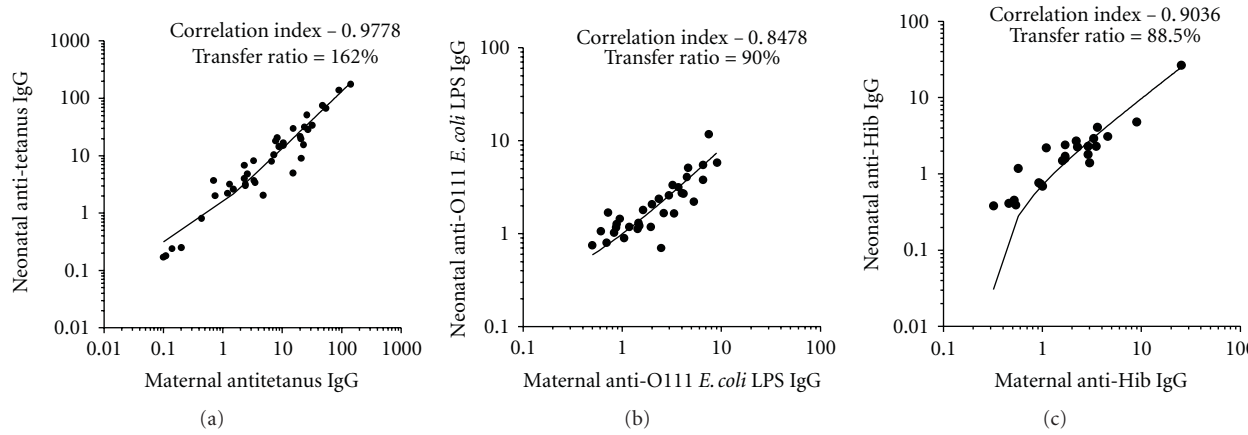

Figure 2: Correlation indexes and placental transfer ratios of maternal and term cord blood IgG levels reactive with tetanus toxoid, O111 LPS from enteropathogenic *E. coli* and Hib polysaccharide. Correlation indexes and placental transfer is higher to thymus-dependent antigens, as tetanus toxoid than to thymus-independent antigens type I and II, as LPS and polysaccharides, respectively.

to the neonate for both total IgG and, interestingly, IgG specific to measles, LPS and other antigens [41, 45, 46].

Since the 1970s, Mäntyjärvi et al. [47] have demonstrated that neonatal anti-influenza A2 IgG levels on average tends to exceed that of the mother if the maternal level is low or normal. When the mother has a high content of total IgG or of a specific antibody, the neonatal value usually remained below the maternal one. This inverse relationship between the efficiency of placental transfer to the respective maternal level was also demonstrated for herpes simplex virus, tetanus toxoid, streptolysin O, and *S. pneumoniae* [48]. This is an interesting observation, because it is known that placental transport is mediated by the interaction between the Fc portion of IgG and the FcRn receptor, in which the Fab portion of this immunoglobulin is not involved. However, this phenomenon suggests an involvement of antigenic specificity of the antibody for this transport, but further studies are needed to investigate the mechanism involved.

4. IgG Transport Depends on the Subclass

It is not clear why some antibody specificities exhibit different transfer impairments in different studies [49]. A plausible explanation may lie in variation in the IgG subclass responses to different antigens and the different affinities of these subclasses to the IgG-transporting FcRn receptors [50, 51]. Preferential transport occurs for IgG1, followed by IgG4, IgG3, and IgG2, for which the FcRn receptors have the lowest affinity [52] (Figure 3). This has been clearly demonstrated in studies on the transfer pattern of different types of specific IgG antibodies showing peculiarities in this transmission. IgG1 and IgG3 are transferred more efficiently across the placenta than IgG2. Furthermore, the transfer of antibodies against viral proteins and antitoxins of the IgG1 subclass occurs more readily. However, antibodies against encapsulated bacteria (*Haemophilus influenzae, Neisseria meningitidis, and Streptococcus pneumoniae*) in which IgG2 prevails, at least after natural exposure, are transferred less efficiently [53, 54], and an effective transplacental transmission of IgG antibodies reactive with LPS involving the IgG1 and IgG2 subclasses was confirmed in our previous studies [55].

In addition, it has been demonstrated that in term neonates with a low birth weight, all IgG subclasses were transferred with reduced efficiency, but IgG1 and IgG2 subclasses were transferred with significantly less efficiency than IgG3 and IgG4. These results demonstrate that low birth weight is associated with impaired placental transfer of IgG1 and IgG2 subclasses.

Overall, at term, IgG in cord blood has a good correlation with maternal levels, and placental transfer is systematically higher to thymus-dependent antigens (proteins), as tetanus toxoid than to thymus-independent antigens, both type I and II, as LPS and polysaccharides, respectively [56] (Figure 2).

5. IgG Transport Depends on Gestational Age

IgG transfer from mother to fetus begins as early as 13 weeks of gestation, and transport happens in a linear fashion as the pregnancy progresses, with the largest amount transferred in the third trimester [39]. Malek and colleagues [57] demonstrated a continuous rise in IgG levels in the fetal circulation between 17 and 41 weeks of gestation. Fetal IgG concentrations were only 5%–10% of the maternal levels at weeks 17–22 but reached 50% of the maternal concentrations at weeks 28–32. The majority of IgG is acquired by the fetus during the last 4 weeks of pregnancy, and fetal IgG concentrations usually exceed maternal ones by 20%–30% at full term [39]. Interestingly, a sharp increase in cord blood levels occurs after the 36th week of gestation.

At term, dependent on the immunological experience of the mother, placental transfer allows the newborn to acquire different specificities of IgG antibodies, resulting in an identical recognition pattern of antigens between the mother and her offspring. As shown in Figure 4, an immunoblotting assay demonstrates identical patterns of enterohemorrhagic *E. coli* (EHEC) antigen recognition between paired mother

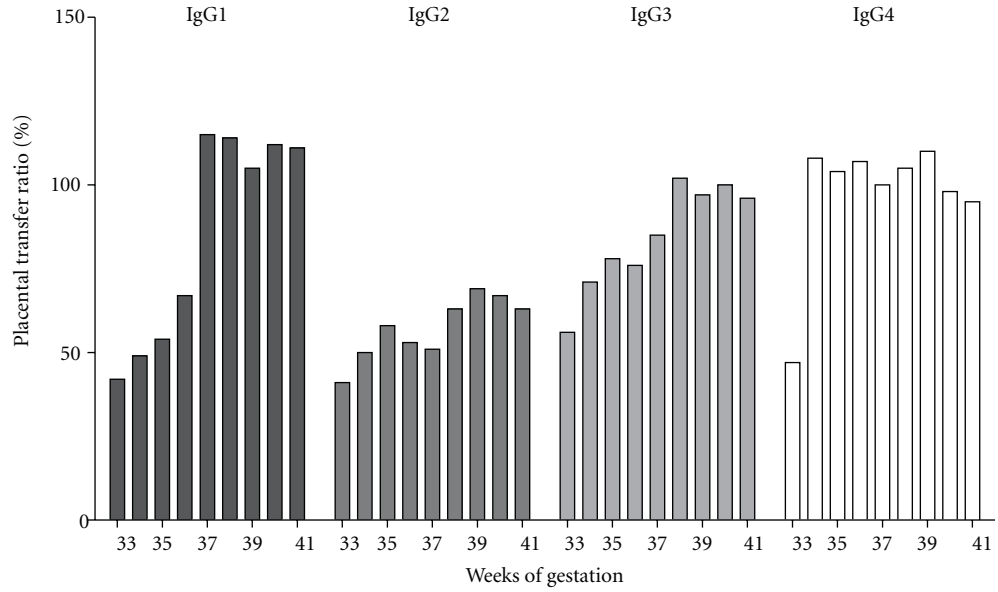

Figure 3: Percentages of placental transfer ratios of IgG subclasses delivered to preterm and term newborns in different gestational weeks. IgG1 and IgG3 transfer ratios rose with increasing gestational age, with IgG1 showing a peak transfer ratio at 37 weeks of pregnancy. IgG2 transfer ratios are always lower than the other IgG subclasses [52].

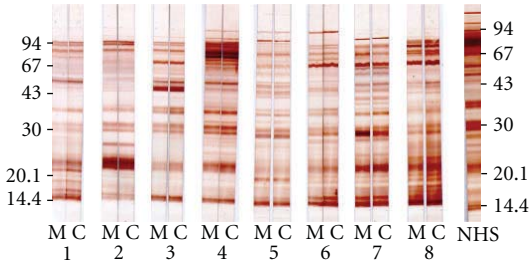

Figure 4: Immunoblotting of anti-EHEC O157:H7 IgG antibodies in the paired maternal and cord samples. Bacterial proteins were separated by 12.5% SDS-PAGE. Paired maternal and cord serum samples are identified numerically. M: maternal serum; C: cord serum; NHS: pool of healthy adult serum samples (normal human serum). The immunoblots were developed with antihuman IgG conjugate. Molecular weight standards are on the left for samples 1–8 and on the right for the pool of normal human serum. It was observed that there is almost complete identity between the antigens recognized by maternal and umbilical cord sera [58].

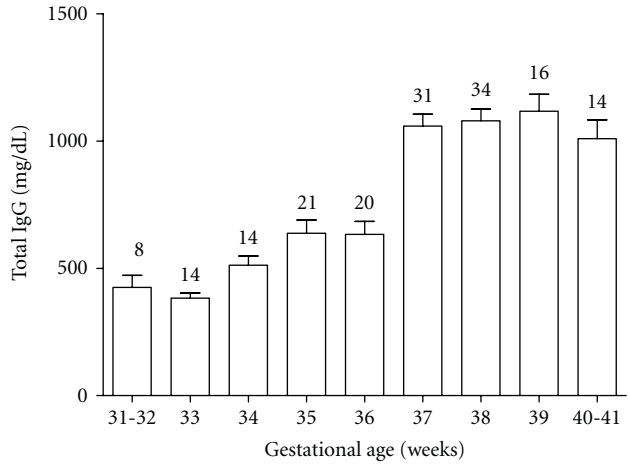

Figure 5: Total IgG concentrations in cord serum samples from newborns in different gestational weeks [46, 60]. *Number of samples in each period.

and term cord sera, thus confirming abundant transfer of the maternal antibody repertoire to the newborn at least for protein antigens.

Maternal age, weight, parity, and type of delivery do not influence placental antibody transfer [59].

Total IgG concentrations in newborns, therefore, are directly related to length of gestation, and infants born at less than 33 weeks of gestation have substantially lower IgG levels than full-term babies (Figure 5). As the expression of FcRn receptor is dependent on gestational age and seems to be more highly expressed in the third trimester of human pregnancy, a reduced placental transfer of antibodies is observed at early stages of gestation. This fact results in a reduced transfer of IgG subclasses, especially IgG1 and IgG2, in preterm compared with full-term babies [56].

Accordingly, in a recent study, van den Berg et al. [54] found significantly lower transplacental transmission of IgG in preterm infants (<32 weeks) than in full-term infants for antibodies against diphtheria, tetanus, pertussis, *Haemophilus influenza* type b (Hib), and *Neisseria meningitides* serogroup C. In agreement with these data, Silveira Lessa et al. [46] evaluated the placental transfer ratios of IgG antibodies reactive with *Klebsiella*, *Pseudomonas,* and *E. coli* O111, O26, and O6 lipopolysaccharides and showed lower anti-LPS IgG transfer ratios in preterm groups (<33 weeks and >33 weeks) as compared with term ones (>37 weeks).

6. The Influence of Maternal Immunization

A main focus of the study of IgG transport across the placenta is maternal vaccination. Currently, several routine vaccines are recommended for pregnant women, such as tetanus toxoid vaccine and inactivated influenza virus vaccine. Others are used in special circumstances, including polysaccharide vaccines, such as pneumococcal polysaccharide vaccine and meningococcal polysaccharide vaccine, and inactivated viral vaccines, such as hepatitis A and B, rabies virus, or inactivated poliovirus vaccines [61]. All of these vaccines are given to protect women from serious diseases during pregnancy and the postpartum period while potentially providing benefits to the fetus and neonate due to placental transmission of those maternal antibodies.

However, many factors may limit the placental transfer efficacy after maternal vaccination, such as the timing between immunization at the pregnancy and delivery, the gestational age of the fetus at birth, total maternal IgG levels and the maternal vaccine-specific IgG and IgG subclasses concentrations [62, 63].

Several randomized studies have been conducted aiming to study the effectiveness of maternal vaccination, targeting important pathogens in early childhood [64, 65]. Prospective studies have demonstrated higher cord antibody levels to influenza in babies born to mothers immunized during pregnancy [66]. Infants of vaccinated mothers were 45%–48% less likely to have influenza hospitalizations than infants of unvaccinated mothers [67]. Maternal influenza vaccination effectiveness for both mother and newborn was also demonstrated in developing countries [68, 69].

Recently, it was showed that Tdap (tetanus and diphtheria toxoids and acellular pertussis antigens) vaccination in pregnancy was safe and significantly increased antibody titers against those antigens. These data reinforced that maternal Tdap vaccination in the second trimester may prevent neonatal pertussis disease in the first 5-6 months of life until infants receive active vaccinations with Tdap at 2, 4, and 6 months of age and establish active immunity [70].

In clinical studies, several factors can affect the transport of antibodies specific to vaccine antigens, particularly the type of vaccine administered. Vaccines with the ability to induce higher maternal levels of IgG and specifically IgG1, such as Hib polysaccharide- (PRP-) conjugate vaccines result in increased concentrations of IgG1 delivered to the fetus [61] and significantly more PRP-specific IgG antibodies during at least the first 2 months of life in diverse populations [71–75]. The same was observed for type III capsular polysaccharide of Group B Streptococcus (GBS) conjugated to tetanus toxoid vaccine, demonstrating an efficient transport of GBS-specific IgG antibodies to the neonate [76].

Reports on maternal-fetal transfer of antibodies against the capsular polysaccharides of *S. pneumoniae* have demonstrated that even term infants generally receive only a fraction (50%–85%) of either naturally acquired [77] or polysaccharide vaccine-induced antibodies from their mothers [63, 78–82]. Although higher IgG antibody levels are found in offspring of immunized compared to unimmunized women, these titers are not maintained for a long time after birth, they likely increase protection from invasive pneumococcal disease until around 120 days after birth, when disease rates are very high [78]. In contrast to the polysaccharide vaccine, maternal immunization schedules including a conjugate pneumococcal vaccine have the potential advantage of stimulating a larger quantity of antibodies of the IgG1 rather than the IgG2 subclass [83].

Another point that merits discussion is that higher doses of passively acquired antibodies may suppress antibody responses to active immunization in early infancy. Several studies have also reported that maternal antibodies can inhibit infant responses to measles, tetanus, whole cell pertussis, and Hib vaccines; this effect varies considerably between different vaccines and studies [84–87]. Regarding toxoids, it was observed that infants who had considerable levels of pre-existing antibodies exhibited lower responses after active immunization to diphtheria toxoid after the second dose, but after 12 months of life, antibody titers do not differ between those infants whose mothers had low titers. For the conjugate PRP-T vaccine, the anti-Hib antibody response was not affected by high maternal antitoxin titers; however, the infants' response to tetanus toxoid was dampened by these high titers. Despite this, all infants achieved protective levels of tetanus antitoxin-IgG after the booster dose with PRP-T. Regarding polysaccharide vaccines, studies have shown no difference in immune response of infants whose mothers received the vaccine or not during pregnancy when they are given the doses at 6–8 months of life. This observation was made with both meningococcal polysaccharide and Hib vaccines [88].

The mechanisms through which maternal antibodies inhibit infant responses to vaccination are not fully understood. However, some plausible explanations are as follows: (i) neutralization of live viral vaccines, (ii) vaccine antigen immune complexes inhibiting infant B cell activation mediated by FcγRIIb receptor, (iii) effective elimination of vaccine antigen coated with maternal IgG antibodies via Fc-dependent phagocytosis, and (iv) vaccine antigenic epitopes being masked or hidden by maternal antibodies, preventing binding by infant B cells [84, 89]. Although persistence of maternal antibodies may limit infant antibody responses, induction of T-cell responses remain largely unaffected by these passively transferred antibodies, because the administration of repeated vaccine doses, as routinely performed for diphtheria-tetanus-pertussis-polio and Hib vaccines, is often sufficient to overcome inhibition by maternal antibodies [90].

7. Placental Transport of IgG in Infectious Diseases

It is well known that antibody transport during pregnancy can be affected by a number of factors and clinical conditions, including placental abnormalities, total IgG concentration in maternal blood, the gestational age of the fetus at birth, and maternal pathologies, such as hypergammaglobulinemia, HIV infection, and placental malaria [91–93]. In addition, preterm labors and intrauterine growth retardation

are associated with a number of pathologies, such as chronic hypertensive disease or hypertensive disease during pregnancy, preeclampsia, gestational diabetes, and infections whose influence in maternal antibody levels is still unknown [94].

In cases of maternal HIV infection or placental injuries, like malaria, a great decrease in antibody transfer has been reported [48, 75, 95–97]. A multivariate regression analysis study determined that placental malaria or maternal HIV infection, independent of maternal hypergammaglobulinemia, are conditions that affect placental transfer of antibodies, and if the mother also has high IgG serum levels, placental transfer is even more impaired [92].

It has been recently demonstrated that HIV-exposed but uninfected infants have reduced transplacental transfer of Hib-, pertussis-, pneumococcus-, and tetanus-specific antibodies than their non-HIV exposed peers. These findings were consistent with two other studies in HIV-infected women from Kenya, indicating that maternal HIV is associated with lower tetanus and measles-specific antibodies in cord blood and also with reduced placental antibody transfer [98, 99]. However, although prenatal HIV exposure was associated with lower specific antibody levels in exposed uninfected infants compared with unexposed infants at birth, after 16 weeks of life, robust and significantly higher antibody responses to pertussis and pneumococcus following routine vaccination were observed in the group of exposed uninfected infants compared with control infants. Therefore, HIV exposure is associated with a greater change in antibody levels between birth and 16 weeks [100].

8. Placental Transfer in Mothers with Primary Immunodeficiencies

Women with humoral deficiencies are dependent on exogenous administration of lgG to prevent recurrent infections with possible severe morbidity and even mortality. In addition, in the absence of the intravenous immunoglobulin (IVIG) therapy, their fetuses may also have an increased risk of infection during intrauterine life and during the first few months after birth because of reduced transplacental transfer of immunoglobulins from those mothers to their offspring [101].

Common variable immunodeficiency (CVID) is not an extremely rare disorder, and currently, many patients reach childbearing age in reasonably good health and become pregnant. CVID represents a heterogeneous group of immunologic disorders, characterized by reduced serum immunoglobulin levels and impaired antibody responses, with variable T cell numbers and function [102]. Its genetic heterogeneity has been studied in the last few years, with the identification of underlying defects in the following genes: ICOS (inducible costimulator), BAFF-R (B-cell-activating factor receptor), TACI (transmembrane activator and calcium-modulator and cyclophilin ligand interactor), CD19, and, more recently, CD20 and CD81 deficiencies [103].

There are only a few reports on total immunoglobulin placental transfer in those cases [104–106], but it was recently shown that CVID mothers under IVIG therapy efficiently transferred exogenous IgG through the placenta in similar patterns as endogenous immunoglobulins, as demonstrated by the following: (i) cord blood IgG levels in term babies were even greater than in the mothers, (ii) a preferential transfer of IgG1, IgG3 and IgG4 compared with IgG2, (iii) antiprotein IgG antibody levels equivalent to or higher than maternal ones in cord serum and good transfer of antipolysaccharide IgG antibodies, and (iv) similar anti-*S. pneumoniae* avidity indexes between mothers and their respective neonates (Table 1) [107]. Thus, CVID patients must be informed about the relevance of regular IVIG administration during pregnancy not only for their own health but also for the immunity of their immature offspring.

9. Placental Transfer in Mothers with Autoimmune Diseases

There are circumstances in which placental transmission of antibodies is detrimental to the neonate. Neonatal lupus erythematosus (NLE) is a rare disease considered to be the exemplary prototypic model of passively acquired systemic autoimmune disease [108]. Maternal IgG autoantibodies against Ro/SSA and/or La/SSB or, less commonly, to U1-ribonucleoprotein (U1-RNP), are transported through the placenta and harm the fetus by causing injury to the skin (cutaneous rash). One of the strongest clinical associations is the development of congenital heart block, which is most often of third-degree severity in a structurally normal heart. This abnormality is an alarming prospect facing 2% of mothers with these autoantibodies [109]. The risk of having a second baby with NLE among women who have already had a baby with NLE increases to 15% [110].

Sera of patients with autoimmune disorders contain an active idiotypic-anti-idiotypic network, which can also be induced in experimental animals following immunization with B-cell epitopes of autoantigens. It has been shown that sera of pregnant women with anti-La/SSB autoantibodies who carry a healthy baby have significantly higher levels of anti-idiotypic antibodies to anti-La/SSB, suggesting that these may serve as protective antibodies for the development of congenital heart block [111]. Therefore, the presence of anti-idiotypic antibodies to autoantibodies against La/SSB may protect the fetus by blocking pathogenic maternal autoantibodies.

The transference of autoantibodies was also reported in neonatal pemphigus, which is characterized as a rare transitory autoimmune blistering disease caused by transfer of maternal IgG autoantibodies specific for desmoglein 3 to the neonate when the mother is affected with pemphigus [112]. This disease is clinically characterized by transient flaccid blisters and erosions on the skin and rarely the mucosa. However, by 3 months, IgG antidesmoglein levels in the neonate are within normal limits [113]. Transient neonatal autoimmune diseases have also been reported for myasthenia gravis and antiphospholipid syndrome, and recently, a case was reported of a newborn with transient epidermolysis bullosa acquisita, a chronic, autoimmune bullous dermatosis

Table 1: Serum immunoglobulin levels, specific antibody concentrations and avidity indexes in the maternal and cord serum and cord/maternal serum ratios for IgG antibodies from a mother with CVID.

	Maternal	Cord	Placental Transfer ratio (%)
Total Immunoglobulin Concentrations			
IgG (mg/dL)	473.0	912.0	190
IgM (mg/dL)	<6.0	12.0	–
IgA (mg/dL)	<3.0	<3.0	–
IgG1 (mg/dL)	362.0	752.0	210
IgG2 (mg/dL)	249.0	192.0	80
IgG3 (mg/dL)	10.0	20.0	200
IgG4 (mg/dL)	6.0	21.0	350
Specific IgG Antibodies Levels			
IgG anti-tetanus toxoid (UI/mL)	1.6	3.1	190
IgG anti-Hib PRP* (mg/L)	3.8	3.7	100
IgG anti-PS§1 (mg/L)/avidity (M)+	2.3/>3.0	2.6/>3.0	110
IgG anti-PS3 (mg/L)/avidity (M)	2.4/>3.0	2.8/>3.0	120
IgG anti-PS5 (mg/L)/avidity (M)	7.3/2.7	8.0/2.7	110
IgG anti-PS6 (mg/L)/avidity (M)	6.9/2.7	6.6/>3.0	100
IgG anti-PS9 (mg/L)/avidity (M)	4.4/2.9	4.7/>3.0	110
IgG anti-PS14 (mg/L)/avidity (M)	12.8/2.5	15.0/2.8	120

–IgM and IgA maternal/cord blood ratios were not performed;
*PRP—polyribosyl–ribitolphosphate polymers;
§Anti-PS—Anti-*Streptococcus pneumoniae* polysaccharide;
+(M)—Avidity index in molarity.

due to the passive transfer of maternal autoantibodies against the noncollagenous terminus of the α chain of type VII collagen [114–116].

In autoimmune diseases in which pathogenic or excess IgG antibodies are the etiological agents, such as myasthenia gravis, bullous pemphigoid, idiopathic thrombocytopenic purpura (ITP), and systemic lupus erythematosus (SLE), it is sometimes advantageous to reduce endogenous serum IgG levels by interfering with FcRn function. One possible way to interfere with the function of FcRn is to overload it with "innocuous" IgG. As FcRn functions as the IgG homeostatic receptor, the level of FcRn expression determines the serum concentration of IgG. Administering large quantities of exogenous IgG raises the serum concentration above this equilibrium set point and saturates FcRn [117]. As a result, the excess IgG that does not bind to FcRn enters the degradative pathway. This results in a shortening of the serum IgG half-life. High-dose IVIG treatment is thought to exert an immunomodulatory effect by numerous mechanisms, including engagement of the inhibitory FcγRIIb receptor [118] and by FcRn saturation [117].

In mouse models of bullous pemphigoid, ITP and autoimmune arthritis, IVIG treatment results in the dilution of pathogenic antibodies to levels beneath the disease-causing threshold [30, 119, 120]. The fact that a therapeutic effect for IVIG is maintained in FcγRIIb-deficient mice and is attenuated in FcRn-deficient mice is strong evidence that an important mechanism of action of IVIG is its ability to compromise FcRn function [30, 121]. This approach provides a valuable tool to prevent neonatal autoimmune disease by exploiting the saturation of FcRn by high doses of IVIG [122–125].

Finally, one interesting point that has been well explored in murine models but not yet in humans is that placental-derived IgG antibodies exert long-life immunoregulatory functions, including imprinting the fetal immune network [126]. Thus, by crossing the placenta, maternal IgG, in addition to providing anti-infectious protection to the infant, could have other active immunoregulatory long-term effects. This mechanism of transplacental antibodies transfer could also be involved in the recognition of allergens and priming of small populations of allergen-specific T cells in the newborn during intrauterine life, which could represent a normal stimulatory signal [127].

10. Conclusions and Perspectives

The maternal IgG antibody transfer varies as a result of total and specific maternal IgG levels, IgG subclass (and thus, the nature of antigen), gestational age, and placental integrity. Knowledge of the features of placental transmission of IgG antibodies is crucial to exploit and manipulate this mechanism to benefit the newborn. The finding that mothers respond well to vaccination and are able to transfer their entire antibody repertoire to their infants is encouraging, raising the possibility of providing protection until the time when the infant is vaccinated. Overall, the employment of IVIG therapy promises to be an area of active research with applications in mothers with primary immunodeficiencies

to promote maternal and newborn protection against infections and in the treatment of various antibody-mediated autoimmune diseases, modulating transfer of harmful autoantibodies.

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Review Article

Cell Death Mechanisms at the Maternal-Fetal Interface: Insights into the Role of Granulysin

Danijela Veljkovic Vujaklija,[1,2] Sonja Sucic,[1,3] Tamara Gulic,[1] Marin Dominovic,[1] and Daniel Rukavina[1]

[1] *Department of Physiology and Immunology, Medical Faculty, University of Rijeka, 51000 Rijeka, Croatia*
[2] *Department of Radiology, Clinical Hospital Center Rijeka, 51000 Rijeka, Croatia*
[3] *Center for Physiology and Pharmacology, Institute of Pharmacology, Medical University Vienna, 1090 Vienna, Austria*

Correspondence should be addressed to Daniel Rukavina, daniel@medri.hr

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During mammal pregnancy, a sensitive balance between hormones, cytokines, humoral factors, and local cellular interactions must be established. Cytotoxic cells infiltrating the decidua are heavily equipped with cytolytic molecules, in particular perforin and granulysin. Granulysin is especially abundant in NK cells which are able to spontaneously secrete high quantities of granulysin. Besides being a potent bactericidal and tumoricidal molecule, granulysin is also found to be a chemoattractant and a proinflammatory molecule. The precise role(s) of granulysin at the maternal-fetal interface has not been elucidated yet. It is possible that it behaves as a double-edged sword simultaneously acting as an immunomodulatory and a host defense molecule protecting both the mother and the fetus from a wide spectrum of pathogens, and on the other hand, in case of an NK cell activation, acting as an effector molecule causing the apoptosis of semiallograft trophoblast cells and consequently leading to various pregnancy disorders or pregnancy loss.

1. Introduction

Pregnancy is a unique event whereby the fetal semiallogenic trophoblast cells develop a close contact with the mother's fully competent immune system. In order for implantation to be successful, the uterus must undergo specific tissue transformation to establish a sensitive cytokine and hormonal balance [1]. Decidualization of the human endometrium following embryo implantation is normally associated with massive recruitment of distinct $CD56^{bright}CD16^{-}$ natural killer (NK) cells [2, 3]. Sharing common cytotoxic pathways with cytotoxic T lymphocytes (CTL), these cells collectively play a vital role in the maintenance of pregnancy and protection against numerous pathogens. There are two main pathways of lymphocyte-mediated cytotoxicity: (i) granule exocytosis pathway and (ii) death receptor signaling pathways [4, 5].

2. Granule Exocytosis Pathway

The granule exocytosis pathway is mediated *via* release of cytotoxic granules directly into the synapse between the effector and their target cells. The granules released contain a pore-forming protein, perforin, an apoptotic/cytolytic protein, granulysin, and a family of serine proteases, granzymes [5–7]. Upon release, perforin inserts itself into the plasma membrane of the target cells, polymerises, and subsequently forms cylindrical pores to allow granzymes, in particular granzyme B, and granulysin to enter the cell and initiate apoptosis [7]. Perforin is essential for the release of granzymes from the cytolytic granules although, as for granulysin, perforin is not necessary for their entry into the target cell [8, 9] (Figure 1-I(A)).

The cytolytic machinery of decidual lymphocytes is optimally primed to kill, but at the same time, it is precisely

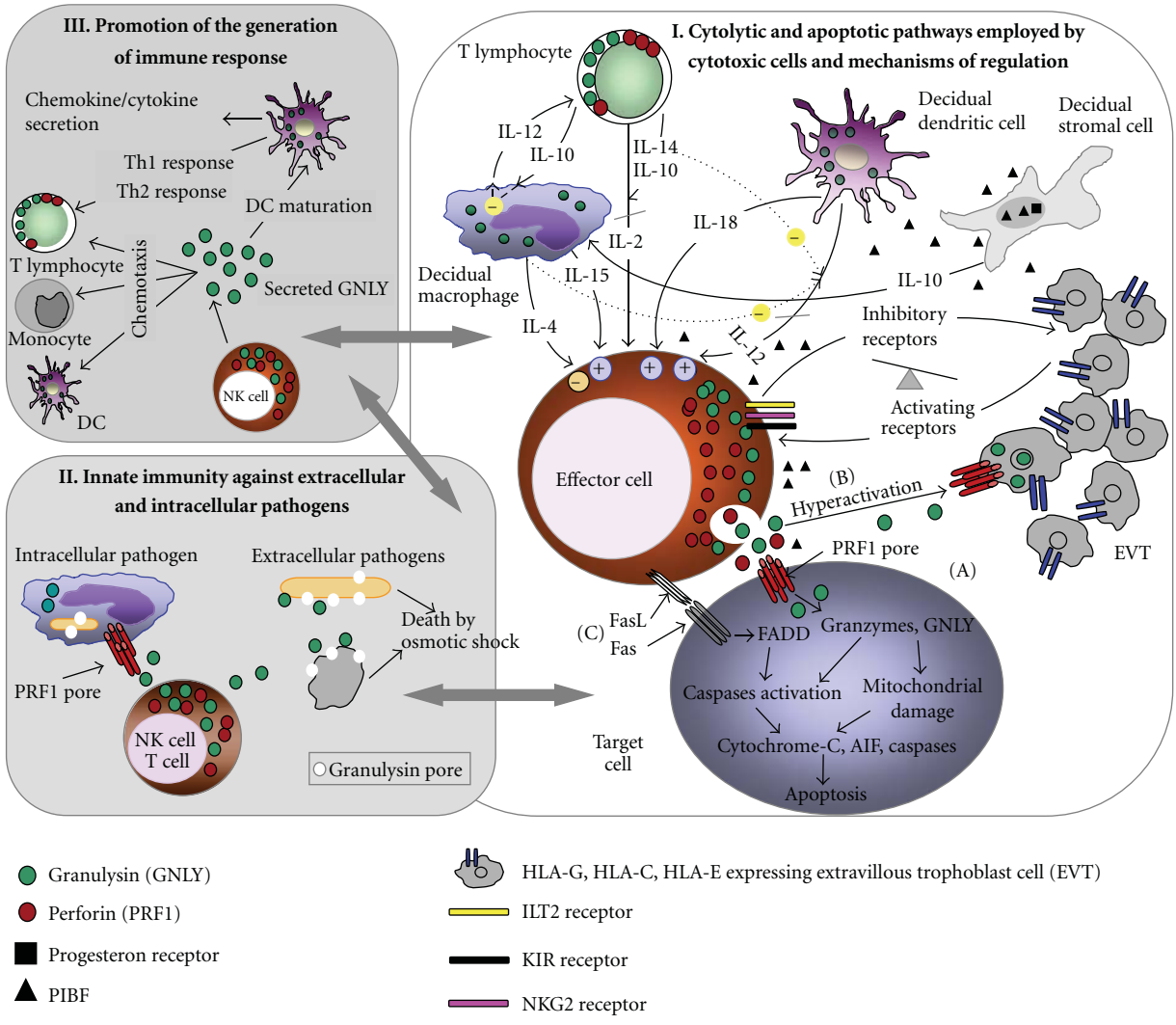

Figure 1: Cytolytic and apoptotic pathways at the maternal-fetal interface employed by cytotoxic cells and mechanisms of regulation with the particular insight to the role(s) played by granulysin.

regulated by humoral factors, cytokines (predominantly Th2 over Th1 cytokines), and local cellular interactions, to prevent accidental activation that could lead to the termination of pregnancy [10, 11]. More than 90% of all decidual $CD56^{bright}CD16^{-}$ cells express perforin, which is present here in higher levels than in any other tissue under physiological or pathological conditions [12]. Nonstimulated decidual NK cells are not cytotoxic, but following an *in vitro* stimulation with Th1 cytokine interleukin (IL)-2 which is in early pregnancy decidua virtually absent, they acquire lymphokine-activated killing (LAK) activity against trophoblast cells [13, 14]. Earlier results from our laboratory support these findings, showing that IL-2 increases perforin gene and protein expression in decidual lymphocytes [4]. Cytolytic activity of decidual lymphocytes is also upregulated by IL-15, IL-18, and IL-12, all acting by increasing perforin and FasL expression [4, 15–17] (Figure 1-I(A), (B)).

Conversely, perforin expression in decidual lymphocytes is downregulated by progesteron, either directly or *via* progesterone-induced blocking factor (PIBF) [10, 18]. The lack of cytolytic activity of decidual NK cells against trophoblasts could also be attributed to inhibitory interactions of nonclassical class I HLA molecules (HLA-G and HLA-E) and low polymorphic classical class I HLA molecule HLA-C expressed by extravillous trophoblast cells with their respective inhibitory receptors immunoglobulin-like transcript 2 (ILT2), C-type lectin family receptor CD94/NKG2A, and killer Ig-like receptors (KIRs) expressed on NK cell surface [19–21]. In normal early pregnancy, specific interaction between NKG2A and HLA-E mediates a dominant negative signal *in vivo*, to prevent perforin recruitment and potentially harmful cytotoxicity [22]. However, decidual NK cells also express a series of activating receptors, such as NKG2D, but also natural killer cytotoxicity receptors NKp30, NKp44, and

NKp46, whose overengagement could trigger decidual NK cell cytotoxic activity [22–24].

3. Death Receptor Pathway

The death receptor pathway is mediated by Fas/FasL, tumor necrosis factor, (TNF-) receptor family, and TNF-related apoptosis-inducing ligand (TRAIL). Fas receptor is ubiquitously expressed within most healthy cells, including NK cells, activated T and B lymphocytes, and dendritic cells [25, 26]. FasL is in human pregnant uterus stored in the intracellular granules of T and NK cells, localized to the sites of close contact with the placental tissue, and can be exocytosed upon nonspecific activation of these cells [27, 28] (Figure 1-I(C)). The ligation of Fas and FasL results in a classical caspase-dependent apoptosis involving the adaptor molecule Fas-associated death domain (FADD) protein [6] (Figure 1-I(C)). In the villous part of the placenta where the Fas/FasL interaction seems to be involved in the regulation of placental growth, FasL is mostly located on cytotrophoblast cells, whereas syncytiotrophoblast cells express low FasL levels. In contrast, interstitial trophoblast cells, which are in close contact with the maternal leukocytes, strongly express FasL, but not Fas. The abundant expression of FasL on extravillous trophoblast probably serves as a defense mechanism against activated maternal leukocytes [4, 29]. Th1 cytokines, IFN-γ and TNF-α, promote Fas expression on trophoblast, thus making them susceptible to Fas/FasL-mediated apoptosis by activated maternal lymphocytes, whereas Th2 cytokines increase the resistance of trophoblast cells to Fas-mediated apoptosis [30]. TRAIL is a TNF-protein family member, structurally and functionally similar to FasL. It is expressed by syncytiotrophoblast and, along with FasL, is likely involved in the maintenance of immunoprivileged conditions at the maternal-fetal interface [31, 32]. The exact role of perforin and death receptor interactions during human pregnancy has not been elucidated yet, but it is believed that they serve as a molecular weapon of uterine NK cells by (i) playing an important role in the acceptance of the fetus and the control of trophoblast invasion and (ii) being crucial for defense against microbe-infected, stressed, or malignant cells.

4. Granulysin-Mediated Cell Death

Another protein that could be vital for the maintenance of normal pregnancy is granulysin. Granulysin is a recently discovered protein, found to be highly expressed in human NK cells and activated CTLs. Over the last decade, granulysin has been a protein of significant scientific interest, mainly due to the cytolytic activity it exhibits on the numerous microbes ranging from extracellular and intracellular bacteria to fungi and parasites, but also due to its tumoricidal activity [9, 33].

The granulysin protein can be found in two forms: 9 and 15 kDa [34]. The cytolytic 9 kDa form is active, and it is achieved *via* a posttranslational proteolytic cleavage at the amino and carboxyl terminals of the 15 kDa precursor granulysin protein [35]. However, Chung and colleagues [36] have recently shown *in vitro* cytotoxic effects even for the 15 kDa granulysin protein. Granulysin protein is expressed in various cell subsets, such as the activated $CD4^+$ and $CD8^+$ T lymphocytes, NK cells and in activated, but not in resting, CTLs [37], NKT cells [38] as well as decidual $\gamma\delta$ T cells [39], and tuberculosis-specific Vγ9/Vδ2 T cells [40].

Granulysin belongs to the family of saposin-like lipid binding proteins, with the highest level of homology to NK-lysin. The three-dimensional structure obtained from X-ray crystallography of recombinant granulysin reveals that it is composed of five α-helices, separated by short-loop regions [41, 42]. It is believed that the lytic activity of granulysin can be explained by its cationic ampholytic structure based on which granulysin disrupts bacterial membrane which generally contains negatively charged lipids and hence mediates bactericidal activity by osmotic shock [43, 44] (Figure 1-II), In contrast, granulysin does not permeabilize target cell membranes when bound to lipid rafts or phospholipid membranes with eukaryotic lipid composition, but it can be internalized into such cells (e.g., infected eukaryotic cells) by lipid rafts and delivered to the early sorting endosomes which afterwards fuse with bacteria-containing phagosomes, where finally the lysis of bacteria is induced [45–47]. The data on the role of perforin in granulysin uptake in infected eukaryotic cells is inconclusive. Stenger et al. [9] reported that in order to kill intracellular pathogens, granulysin requires perforin as a cofactor to enter the host cells, whereas Walch et al. [47] report that perforin promotes granulysin-mediated bacteriolysis not by the formation of stable pores that allow passive diffusion of granulysin but rather by an increase in endosome-phagosomes fusion triggered by an intracellular Ca^{2+} rise (Figure 1-II).

Death of the tumor cells is caused by granulysin-initiated apoptosis. Upon binding to the membrane based on charges, granulysin activates Sphingomyelinase followed by a slow increase in ceramide concentration or induces an increase in intracellular calcium and efflux of intracellular potassium [48–50]. Both pathways are linked with fast mitochondrial membrane damage, which is the key step in granulysin-induced apoptosis [51, 52]. This results with release of cytochrome C and apoptosis-inducing factor A [53] followed by activation of caspases and endonucleases and finally the cell death by apoptosis [49, 54] (Figure 1-I(A)). The latest report by Saini et al. [50] shows that NK cell-delivered granulysin and recombinant granulysin induce target cell death through distinct pathways. Granulysin delivered by NK cells does not cause mitochondrial damage or activates either caspase-3 or caspase-9 in target cells, whereas recombinant 9-kDa granulysin activates these pathways. Unlike recombinant granulysin, NK cell-delivered granulysin causes both endoplasmic reticulum stress and caspase-7 activation in target cells [50].

Recently, granulysin has been characterized as the first lymphocyte-derived protein found to act as an alarmin, capable of promoting antigen-presenting cell (APC) recruitment and antigen-specific immune response [55]. At nanomolar concentrations, predominantly 15 kDa granulysin acts as a chemoattractant for immune cells, such as monocytes, NK cells, and $CD4^+$ and $CD8^+$ memory T-lymphocytes

[55, 56] and has a proinflammatory effect due to its ability to activate monocytes to produce cytokines, such as IFN-γ and TNF-α, and chemokines, such as MCP and RANTES [56] (Figure 1-III). The latest results by Castiello et al. [57] revealed that *in vitro,* 15 kDa granulysin induces immune response, chemotaxis, and cell adhesion genes in human peripheral blood monocytes. Further, granulysin-treated monocytes upregulated genes involved in the activation of pathways related to fundamental dendritic cell functions, such as costimulation of T-cell activation and Th1 development, for example, upregulation of genes in the IL-12 and STAT4-dependent signaling [57].

Therefore, granulysin presents a novel cytolytic molecule with immense biomedical and therapeutic potential [58]. Besides its ability to kill bacteria, fungi, and parasites, granulysin can block viral replication and trigger apoptosis in infected cells [59]. The fact that it can kill many deadly pathogens makes granulysin, and its recombinant peptide derivatives, an attractive target in the development of novel classes of antibiotics, with less resistance observed for most currently available therapies. In addition, 15 kDa granulysin holds promise for therapeutic applications aimed at the activation of the immune response.

5. Granulysin at the Maternal-Foetal Interface: A Double-Edged Sword

Granulysin mRNA is expressed in human endometrium of nonpregnant women with highest expression in the late secretory phase of the menstrual cycle, correlating with the increase in uterine NK cell number towards the end of the secretory phase [60]. Granulysin expression is most likely under the control of progesteron since the antiprogestin treatment of secretory endometrial explants decreased the granulysin mRNA expression [60]. Perforin exhibits a similar pattern of expression during menstrual cycle with massive recruitment of perforin-positive cells following the progestin-induced endometrial decidualization [61]. The expression of granulysin mRNA in early pregnancy and second trimester placentas increases further, whilst its expression is downregulated at term (37–40 weeks) placentas [62].

The granulysin (GNLY) gene expression in secretory phase human endometrium was localized to uterine NK cells scattered around the stroma and surrounding the glandular epithelium [63]. Our recent results show that first trimester pregnancy decidual tissue is diffusely infiltrated by granulysin-positive cells which accumulate around uterine glands and blood vessels [64, 65]. Granulysin protein was also found in peripheral blood lymphocytes of pregnant women but in significantly lower amounts [64]. Immunophenotipisation of first trimester pregnancy decidual lymphocytes revealed that over 85% of $CD56^+CD3^-$ NK cells and 75% of $CD56^+CD3^+$ NKT cells express granulysin. Similar to decidual, peripheral blood NK and NKT cells also highly express granulysin. At gene level, granulysin is highly expressed in all decidual lymphocyte subpopulations, especially in $CD56^+$ cells. In comparison to other cytolytic mediators (perforin, FasL, TRAIL), granulysin mRNA is present in significantly higher levels in $CD56^+$ and $CD56^+CD3^+$ cells, but in T lymphocytes only, no differences between granulysin and perforin mRNA levels were observed (unpublished data, manuscript in preparation). It is known that NK cells constitutively express high levels of granulysin, whereas granulysin expression in T lymphocytes is inducible after mitogen or alloantigen stimulation [66, 67]. Surprisingly, more than half of decidual T lymphocytes (approximately 58%) express granulysin protein, whereas only few percent of peripheral blood T lymphocytes express granulysin [64]. The striking increase of the percentage of granulysin-positive decidual T lymphocytes suggests that these cells are locally activated, even in the early phase of pregnancy, probably after the contact with semiallogenic fetal cells and mature dendritic cells. Hence, these cells could play a protective role against different pathogens at the maternal-fetal interface by delivering granulysin which in turn either reduces the viability of the extracellular or intracellular pathogens as described previously, possibly leaving the host cell intact [9, 45]. Moreover, Stenger et al. [9] correlated the CTLs ability to reduce the viability of intracellular pathogens with granulysin expression in these cells. The protective role of granulysin was further supported by findings by Fleming and coworkers [60] which stated that granulysin enhances the innate immune capacity of endometrium since women taking the oral combined hormonal contraceptive pill or wearing a levonorgestrel intrauterine system have a significantly lower expression of granulysin mRNA in the late secretory phase of menstrual cycle and are more susceptible to various infections. Granulysin was also found to be expressed by $\gamma\delta$ T cells of human early-pregnancy decidua, further supporting the protective role of granulysin as an innate immunity molecule in early pregnancy [39].

Granulysin could also have a very important immunomodulatory role at the maternal-fetal interface (Figure 1-III). Recently, Tewary et al. [55] reported that both 9 kDa and 15 kDa forms of granulysin induce the migration as well as functional and phenotypical maturation of human monocyte-derived dendritic cells (Mo-DC) by upregulation of CD80, CD83, and MHC class II molecules. Also, it induces higher production of IL-6, IL-8, IL-12, IL-10, and TNF-α by Mo-DC and enhances their capacity to stimulate allogeneic T-cell proliferation *in vitro* [55]. The 15 kDa granulysin might also play an important role in activating the immune system in response to pathogens by inducing monocytes to recruit other immune cells by upregulating a wide group of genes responsible for chemotaxis of different immune cells, such as neutrophils (CXCL1, CXCL3), T cells (CXCL11, CXCL12, CCL20, and CCR7), monocytes (CCL2, CCL20), macrophages, and dendritic cells (NRP2, SEMA3A) [57]. Cognition that granulysin acts as a chemoattractant to such various cells types opens the question whether granulysin produced by decidual NK cells could also mobilize invasive trophoblast cells.

Our latest results revealed that uterine $CD56^+$ cells spontaneously secrete high levels of granulysin [64]. The amounts of granulysin secreted by decidual $CD56^+$ cells were almost equivalent to values at which the peak migratory

response of dendritic cells was observed in the study by Tewary et al. [55]. In contrast, peripheral blood $CD56^{+}$ cells virtually do not secrete granulysin after 2 hrs in culture. After 18 hrs in culture, they start secreting very low levels of granulysin, which are well in agreement with studies by Ogawa et al. [68] and Sakai et al. [69]. In our opinion, considering that the female reproductive tract is potentially exposed to wide range of pathogens, and infections of genital tract during pregnancy may have severe consequences such as miscarriage or preterm birth, the spontaneous secretion of high levels of granulysin probably serves as a mechanism of protection of both mother and fetoplacental unit [64, 70, 71]. After being spontaneously secreted into local environment, granulysin might be able to promptly kill various pathogens without causing significant damage to normal cells, whereas simultaneously through its effects on APCs and other immune cells it might participate in the regulation of the adaptive immune response (Figures1-II and 1-III). Moreover, decidual APCs, both dendritic cells, and macrophages, and IL-15 which is known to be produced by APC, contribute to the maintenance of high granulysin expression in decidual NK cells, thus providing continuous local protection [64]. Another important role of granulysin could be in one of key events of early pregnancy, the angiogenesis, since Langer and coworkers demonstrated that NKG5 protein (granulysin) secreted by decidual NK cells and activated T lymphocytes stimulates mitogenicity of endothelial cells and may be involved in angiogenesis [72].

On the other hand, several studies have shown that granulysin may be involved in different pregnancy disorders [69, 73]. Granulysin was previously associated with the development of preeclampsia as a Th1 marker. Its serum levels in preeclamptic patients were well associated with the percentage of peripheral blood Th1 cells, Th1/Th2 ratios, and the clinical outcome [69]. In cases of ectopic pregnancy, granulysin-expressing cells were virtually absent in the tubal mucosa which could explain invasiveness of trophoblast cells. Interestingly, the uterine decidua of ectopic pregnancy was simultaneously heavily infiltrated with granulysin-positive cells [65].

As mentioned, during normal pregnancy, uterine NK cells are not directed to kill trophoblast cells, but in case of excessive Th1 response due to infection or inflammation, these cells become hyperactivated and potentially cytotoxic (Figure 1-I(B)) [30, 74]. Nakashima et al. [73] have shown that granulysin contributes to apoptosis of extravillous trophoblast cells in spontaneous abortions. Granulysin secreted by $CD56^{bright}$ uterine NK cells was detected in the cytoplasm and nuclei of apoptotic extravillous cytotrophoblast cells suggesting that granulysin may be a key substance in induction of spontaneous abortion. *In vitro* studies showed that only hyperactivated, that is, IL-2 stimulated uterine NK cells secrete granulysin which in turn actively accumulates in nuclei of extravillous trophoblast cell line. Granulysin entrance into trophoblast cells, as well as NK cell-mediated killing of target tumor cells, was shown to be dependent on perforin and cell-to-cell contact. [50, 73] (Figure 1-I(B)). Several studies have shown that HLA-G molecule inhibits natural killer cell-mediated cytotoxicity [75, 76]. In fact, our latest results show that one of the mechanisms which might prevent an excessive NK cell activation during normal pregnancy could be HLA-G mediated. Namely, decidual NK cells after the contact with HLA-G, transfected NK sensitive K562 cells express and secrete significantly lower quantities of granulysin in comparison to NK cells exposed to HLA-C-transfected K562 cells (unpublished data, manuscript in preparation).

In light of all that is currently known, and considering that granulysin is abundantly expressed in early pregnancy decidua, especially in NK cells, we hypothesize that granulysin is a novel lethal weapon of uterine NK cells acting as a double-edged sword at the maternal-fetal interface (Figure 1). At the same time, it acts as a host defense molecule protecting both the mother and the fetus from a wide spectrum of pathogens, and as an immunomodulatory molecule by inducing chemotaxis of different immune cells, phenotypical and functional maturation of dendritic cells, and by upregulating the adaptive immune response. On the other hand, in case of NK cell activation, along with perforin, granulysin can act as an effector molecule causing the apoptosis of semiallograft trophoblast cells, consequently leading to various pregnancy disorders including spontaneous pregnancy termination, intrauterine fetal growth retardation, and preeclampsia. However, many details regarding the exact mechanisms underlying the remarkable modes of granulysin action and its physiological roles remain yet to be elucidated.

Decidual cytotoxic cells are heavily equipped with cytolytic molecules, in particular perforin and granulysin. Specific interactions between nonclassical class I MHC molecules expressed on extravillous trophoblast (EVT) cells and their inhibitory and activating receptors expressed on NK cell surface, along with antigen-presenting cells and complex network of cytokines and hormones, contribute to the maintenance of pregnancy. Th2 cytokines (IL-4, IL-10), which are predominant in early pregnancy decidua, downregulate cytolytic activity, whereas Th1 cytokines (IL-2, IL-15, IL-12, and IL-18) upregulate the same. In case of activation, the interaction between target and effector cells results in effector cell activation and directed granule exocytosis (IA). In case of hyperactivation of NK cells, that is, stimulation of IL-2, granulysin is released from cytoplasmic granules of NK cells and accumulates in the cytoplasm and nuclei of EVT cells in spontaneous abortions (IB). The ligation of FasL, expressed on NK or T-cell surface, with the death receptor Fas, on the target cell, results in caspase cascade activation (IC). All pathways result in endonuclease activation and apoptosis. At the same time, high granulysin levels might have a protective role at the maternal-fetal interface by (i) contributing to the local innate immune capacity against intracellular and extracellular pathogens (II), or (ii) acting as an immunomodulatory molecule by inducing phenotypical and functional maturation of dendritic cells, and (iii) as a proinflammatory molecule by inducing chemotaxis of different immune cells and upregulating the adaptive immune response (III).

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Research Article

Inflammatory Cytokines in Maternal Circulation and Placenta of Chromosomally Abnormal First Trimester Miscarriages

Jean Calleja-Agius,[1,2] Eric Jauniaux,[1] and Shanthi Muttukrishna[3]

[1] *UCL EGA Institute for Women's Health, University College London, 86-96, Chenies Mews, London, WCIE 6HX, UK*
[2] *Department of Anatomy, Faculty of Medicine and Surgery, University of Malta, Tal-Qroqq, Msida 2090, Malta*
[3] *Anu Research Centre, UCC Department of Obstetrics and Gynaecology, Cork University Maternity Hospital, 5th Floor, Wilton, Cork, Ireland*

Correspondence should be addressed to Jean Calleja-Agius, jean.calleja-agius@um.edu.mt

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The impact of abnormal placental karyotype on the inflammatory response within the villous tissue and peripheral circulation of women with miscarriage was evaluated. Villous ($n = 38$) and venous blood samples ($n = 26$) were obtained from women with missed miscarriage. Tissue chromosome analysis indicated 23 abnormal and 15 normal karyotypes. Concentration of tumour necrosis factor alpha (TNFα), TNF-R1 and TNF-R2, and interleukin (IL)-10 were measured using flowcytometric bead array in fresh villous homogenate, cultured villous extracts, culture medium, maternal whole blood, and plasma. Plasma TNFα/IL-10 ratios were significantly ($P < 0.05$) lower in miscarriages with abnormal karyotype. In the abnormal karyotype group, there were significantly higher levels of TNFα ($P < 0.01$), IL-10 ($P < 0.01$), TNF-R1 ($P < 0.001$), and TNF-R2 ($P < 0.001$) in the villous extracts and culture-conditioned medium compared to normal karyotype group. In miscarriage with abnormal karyotype, there is an exacerbated placental inflammatory response, in contrast to miscarriage of normal karyotype where maternal systemic response is increased.

1. Introduction

Early pregnancy loss is the most common pregnancy complication [1]. Around 60% of first trimester miscarriages are associated with a chromosomal abnormality [2–4]. Most chromosomal abnormalities are associated with primary abnormal trophoblast invasion of the uterine decidua [5–7]. As the placental development becomes increasingly dependent on fetal synthesis towards the end of the first trimester, isolated major fetal structural defects leading to early fetal demise can also lead to secondary placental dysfunction causing a miscarriage [8].

Inflammatory processes taking place at the feto-maternal interface are essential for normal implantation in human pregnancy [9–11]. It has been suggested that the main regulator of this inflammatory reaction could be uterine natural killer (NK) cells [10]. Proinflammatory cytokines like tumour necrosis factor alpha (TNFα) have been shown to inhibit trophoblast migration through the elevation of plasminogen activator inhibitor-1 in first trimester villous explant cultures, causing abnormal trophoblast invasion [12]. TNFα has also been shown to downregulate the endocrine function of trophoblasts, leading to pregnancy failure [13]. It has been suggested that a network might exist in which hormones together with cytokines regulate the decidual expression of HLA-G, an antigen preferentially expressed by trophoblast, thus, maintaining maternal-fetal tolerance [14].

There is evidence of a shift in T-helper 1 (TNFα): T-helper 2 (interleukin, IL-10) ratio in the maternal circulation and placental villous tissue in first trimester miscarriages [15–17]. However, most previous studies have used placental tissue for early pregnancy failure without knowledge of the karyotype or have focused on women presenting with recurrent miscarriage, and thus little is known about the impact of the conceptus karyotype on the placental and systemic inflammatory responses in early pregnancy failure. The aim of this study was therefore to investigate the

maternal circulatory levels, villous expression, and secretion *in vitro* of TNFα, TNFα receptors, and IL-10 and to evaluate the Th1 and Th2 cytokine ratio in early pregnancy failures with and without a chromosomal abnormality.

2. Materials and Methods

Chorionic villous samples (n = 38) were obtained from women presenting with a missed miscarriage, undergoing the evacuation of retained products of conception (ERPC) at University College London Hospital (UCLH). All women were nonsmokers, with normal body mass index (BMI) ranging between 20 and 30, not on medication, and with a history of regular menstrual cycles. The date of the last menstrual period (LMP) was used to calculate the gestational age, and women with a history of recurrent miscarriage or who did not know their LMP were excluded from the study. The gestational ages at the time of ERPC ranged between 9 weeks and 0 days and 13 weeks and 6 days gestation. Ultrasound measurement of the fetal crown-rump length (CRL) was used to evaluate the time interval between fetal demise and the surgical procedure of ERPC.

In 12 cases with normal karyotype and 14 with abnormal karyotype, maternal peripheral venous blood (10 mL) was collected by sterile venepuncture into bottles with lithium heparin at the time of ERPC. One mL of uncoagulated blood was aspirated aseptically for whole blood analysis while the rest was centrifuged within 2 hours of collection, and the plasma supernatant was stored at −80°C until assayed.

This study was approved by the joint UCL/UCLH ethics committee on the ethics of human research. Written informed consent was obtained from each participant at the Early Pregnancy Unit prior to the surgical uterine evacuation of retained products of conception (ERPC).

2.1. Flowcytometric Analysis of Fluorescent Antibody-Labelled Whole Blood. Initial whole blood validation experiments showed that an incubation period of 12 hours with 40 ng/mL of lipopolysaccharide (40 LPS) gave the highest increment above basal level (0 LPS) in terms of cytokine expression by the activated viable monocytes. Dual antibody labelling was carried out with specific mouse antihuman antibodies (AbD Serotec, Oxford, UK) that were conjugated to spectrally distinct fluorochromes to identify the monocyte population (CD14) positive for the cytokine/receptor of interest. The method we used has been described elsewhere [18].

2.2. In Vitro Cultures. Following the ERPC, the placental villi were separated from the rest of the POC and washed twice in sterile Hank's Balanced Salt Solution with 0.1% Gentamycin Solution (Sigma-Aldrich, St Louis, USA) and 1% Amphotericin B (Invitrogen, Paisley, UK) to remove any blood. A biopsy of villous tissue measuring ~1 cm^3 was snap frozen in liquid nitrogen and stored at −80°C until homogenised to measure the cytokine/receptor content in the villous tissue on the day of evacuation (Day 0). In all cases, a villous sample was sent to a commercial cytogenetic laboratory (TDL, London, UK) within 2 hours of the ERPC, and karyotyping was carried out by standard culturing, suspension harvest and G-band analysis methodology [19].

Villous tissue obtained from the ERPC was divided into equal sections under sterile conditions under a laminar hood and weighed. Each of the villous biopsies of known weight was cultured in 24-well culture plates containing 1 mL culture medium per well, in triplicate wells. Villous explants derived from each individual woman were cultured separately for 3 days at 6% and at 20% oxygen (O_2) levels in a moist incubator at 37°C, 5% CO_2. The culture medium was made up of D-MEM: F-12 (1 : 1) + GlutaMAX-I (Invitrogen, Paisley, UK) with 0.1% Gentamycin Solution (Sigma-Aldrich, St Louis, USA) and 1% Amphotericin and 1% Insulin-Transferrin-Selenium A serum supplement (Invitrogen, Paisley, UK).

Villous explant culture conditioned medium and placental villi were collected after incubation for 1 day, 2 days, and 3 days from the individual wells. Cultured villous explants were homogenized for cytokine analysis. Homogenization of villous tissue was carried out as previously described by our group [20]. All cytokines/receptors concentrations in villous explants homogenates and culture-conditioned medium were normalized against weight of tissue/well as pg/gram villous tissue.

2.3. Cytokine and Receptor Assays. Cytokines and receptors in plasma, culture medium, and homogenised villi were measured using BD Cytometric Bead Array (CBA) Human Soluble Protein Flex Sets and BD FACSArray bioanalyzer flowcytometer following manufacturer's instructions (BD Biosciences, San Jose, California, USA). The limit of detection was 0.7 pg/mL for TNFα, 0.13 pg/mL for IL-10, 5.2 pg/mL for TNF-R1, and 1.4 pg/mL for TNF-R2. The intra-assay coefficient of variation was 10.2% for TNFα, 6.4% for IL-10, 2.6% for TNF-R1, and 7.1% for TNF-R2. The interassay coefficient of variation was 5% for TNFα, 11% for IL-10, 10.1% for TNF-R1, and 5.6% for TNF-R2. The results were presented in graphical and tabular formats using the FCAP Array Software (BD Biosciences, San Jose, Calif, USA).

2.4. Statistical Analysis. For villous cytokine expression and the time- and O_2-dependent villous explant medium concentrations, the data were log transformed to achieve normality, which was confirmed using the Shapiro-Wilks test and Q-Q plots. Outliers were identified using Cook's distance, and those exceeding the threshold $4/n$ were removed.

For villous *in vivo* cytokine expression, ANOVA was performed on the log-transformed data, to estimate means for each cytokine by karyotype, adjusted for gestation at sampling. Multivariate linear regressions were carried out on the log-transformed data, to estimate the means for each cytokine by karyotype, O_2 level (6 versus 20%), and day of culture (Day 1, 2, or 3), adjusted for gestation. For cytokine/receptor levels in the plasma and monocytes, data were normalized by log transformation, and unpaired t-test was carried out.

A P-value less than 0.05 was considered statistically significant. The statistical analysis and graphs were produced

Table 1: *In vivo* cytokine and receptors, measured in snap frozen villous tissue collected on the day of ERPC—comparison done using 2-way ANOVA, using the variables of karyotype (normal versus abnormal) and gestation (weeks). There was no significant difference between gestation and karyotype. Estimated marginal means for each marker by karyotype, adjusted for gestation, are presented here.

Cytokine/receptor	Normal karyotype pg/g (95% CI)	Abnormal karyotype pg/g (95% CI)	Normal versus abnormal karyotype *P* value
TNFα	8.2 (5.3, 12.6)	7.2 (4.2, 12.2)	0.92
IL-10	2.2 (1.3, 3.8)	2.9 (1.6, 5.2)	0.79
TNFα/IL-10	4.5 (2.2, 9.0)	2.3 (1.3, 4.2)	0.62
TNF-R1	2499.1 (1488.8, 4195.3)	2753.26 (1927.21, 3933.4)	0.56
TNF-R2	12440.9 (6876.6, 22507.7)	12157.9 (8716.9, 16957.2)	0.68

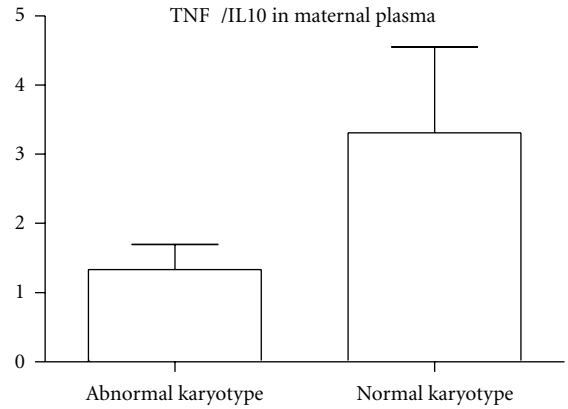

Figure 1: TNFα/IL-10 in the maternal circulation (abnormal karyotype, $n = 14$; normal karyotype, $n = 12$). There was a significantly ($P < 0.05$) lower TNFα/IL-10 ratio in the plasma of miscarriages of abnormal karyotype than in those with normal karyotype.

using SPSS v.17 (SPSS Inc., Chicago, Ill, USA) and GraphPad Prism v.5 (GraphPad Software inc., San Diego, Calif, USA).

3. Results

Karyotyping of the products of conception (POC) indicated 15 with normal karyotype and 23 abnormal karyotypes including 16 trisomies, 4 monosomy X, and 3 triploidies. There was no difference in maternal BMI, age, parity, and ethnic distribution between the group with and without a chromosomal abnormality. The gestation at the time of ERPC, interval in days between estimated fetal demise and ERPC and fetal sex ratio was similar in both groups. In the cases where maternal blood was collected, karyotyping showed 12 normal karyotypes and 14 abnormal karyotypes.

3.1. Maternal Plasma Concentrations. The levels of TNFα, TNF-R1, TNF-R2, and IL-10 were not significantly different between miscarriages with normal and abnormal karyotype. TNFα/IL-10 ratio in the plasma was significantly ($P < 0.05$) lower in miscarriages with an abnormal karyotype than those with normal karyotype (Figure 1).

3.2. Maternal Circulatory Monocyte Concentration. There was no significant difference between intracellular levels of TNFα, TNF-R1, TNF-R2, and IL-10 in the monocytes in plasma samples of both groups. There was a 3-fold higher % LPS stimulation of TNFα in the normal karyotype compared to the abnormal karyotype group (Figures 2 and 3).

3.3. Expression of Cytokine and Receptors in Villous Tissue. No significant differences were found in the cytokine and receptor levels in the snap frozen villous tissue homogenate samples between the groups presenting with normal and abnormal karyotype. These levels were similar in the different gestational subgroups, and across gestation (Table 1).

3.4. Villous Tissue In Vitro Secretion and Content of Cytokines and Receptors. The ANOVA test indicated that gestation has no significant impact on the relationship between cytokine/receptor levels and karyotype in culture conditions at both O_2 concentrations.

Time in culture (1, 2, or 3 days) and O_2 concentration (6 versus 20%) did not affect the mean cytokine/receptor levels or TNFα/IL-10 ratio in villous homogenates or culture-conditioned medium adjusted for gestation (Table 2).

3.4.1. Villous Tissue Homogenates. Significantly higher levels of TNFα ($P < 0.01$), IL-10 ($P < 0.001$), and TNF-R2 ($P < 0.001$ were found in cultured villous extracts in the group with an abnormal karyotype compared to normal karyotype group. There was no significant difference in TNFα/IL-10 (Table 2).

3.4.2. Culture Medium of Villous Tissue Samples. Significantly higher levels of TNFα ($P = 0.001$), IL-10 ($P < 0.01$), and TNF-R1 ($P < 0.001$) were found in the culture-conditioned medium of abnormal karyotype group compared to the normal karyotype group. There was no significant difference in TNFα/IL-10 (Table 2) between the groups.

4. Discussion

The results of our study indicate that, in sporadic miscarriages with a normal karyotype, there is an increased

Table 2: Absolute means and standard error of the mean (SEM) at different oxygen concentrations (6 versus 20%) for villous explant culture experiments for miscarriages with normal karyotype ($n = 15$) and abnormal karyotype ($n = 23$), adjusted for gestation.

(a) Villous explant cultures at 6% Oxygen

Marker (pg/mL/g)	Normal karyotype Day of culture 1		2		3		Abnormal karyotype Day 1		2		3	
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)
TNFα Homo	7.0	(4.0, 12.4)	20.6	(5.3, 79.9)	6.0	(0.8, 44.9)	25.4	(14.1, 45.8)	26.9	(12.2, 59.2)	17.3	(6.7, 44.6)
IL-10 Homo	0.3	(0.2, 0.4)	0.8	(0.4, 1.9)	0.4	(0.2, 0.9)	1.0	(0.6, 1.7)	0.9	(0.4, 2.3)	1.3	(0.6, 2.9)
TNF-R2 Homo	995.9	(469.1, 2114.3)	2334.6	(740.5, 7360.2)	841.6	(274.1, 2584.4)	3159.2	(1804.8, 5530.2)	4243.4	(2901.4, 6206.2)	4279.2	(2045.4, 8952.8)
TNF-R1 Homo	314.0	(174.4, 565.5)	517.5	(252.2, 1061.9)	246.7	(161.4, 377.2)	330.3	(176.9, 616.7)	600.3	(311.3, 1157.6)	278.4	(106.7, 726.2)
TNFα/IL10 Homo	24.7	(14.2, 42.9)	25.5	(4.9, 131.3)	14.7	(4.1, 52.2)	22.0	(12.7, 37.9)	20.0	(10.0, 40.0)	13.4	(7.6, 23.8)
TNFα CM	238.6	(87.3, 651.9)	40.4	(20.4, 80.0)	53.6	(3.3, 863.7)	273.1	(156.1, 477.9)	213.2	(125.5, 362.2)	294.2	(179.1, 483.4)
IL-10 CM	134.1	(11.0, 1628.1)	2.1	(0.1, 41.6)	3.6	(0.1, 929.5)	67.1	(11.1, 404.4)	83.5	(6.2, 1116.6)	37.0	(2.5, 552.4)
TNF-R2 CM	1371.3	(504.9, 3724.8)	2276.1	(827.3, 6262.5)	2712.2	(1972.4, 3729.5)	1109.1	(364.4, 3375.9)	5120.8	(2835.5, 9248.1)	6735.4	(3554.1, 12764.3)
TNF-R1 CM	471.1	(202.5, 1095.9)	430.0	(216.5, 854.0)	472.2	(389.9, 571.8)	645.0	(478.4, 869.6)	1316.6	(882.2, 1964.9)	1186.4	(612.7, 2297.2)
TNFα/IL10 CM	1.8	(0.3, 9.1)	19.2	(1.8, 200.9)	15.0	(0.7, 335.4)	3.6	(0.9, 14.2)	2.9	(0.3, 24.6)	8.0	(0.7, 86.6)

(b) Villous explant cultures at 20% oxygen

Marker (pg/mL/g)	Normal karyotype Day of culture 1		2		3		Abnormal karyotype Day of culture 1		2		3	
	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)	Mean	(95% CI)
TNFα Homo	18.9	(7.2, 50.0)	22.0	(5.0, 95.5)	2.5	(0.7, 8.7)	20.3	(10.1, 40.9)	32.6	(18.2, 58.2)	27.9	(13.4, 57.8)
IL-10 Homo	1.0	(0.4, 2.7)	0.3	(0.2, 0.7)	0.2	(0.1, 0.6)	1.9	(0.9, 3.8)	2.2	(1.0, 5.0)	1.1	(0.5, 2.4)
TNF-R2 Homo	3121.9	(1859.5, 5241.1)	1939.0	(562.8, 6680.3)	1107.7	(202.1, 6070.6)	3713.7	(2408.8, 5725.5)	4967.9	(2460.7, 10029.7)	3789.8	(2045.8, 7020.4)
TNF-R1 Homo	463.1	(218.9, 980.0)	430.6	(179.1, 1035.4)	370.6	(222.5, 617.3)	551.3	(318.5, 954.2)	645.2	(347.8, 1197.2)	738.0	(393.5, 1384.3)
TNFα CM	334.9	(164.4, 681.9)	44.1	(12.5, 174.8)	45.8	(5.1, 411.8)	241.8	(118.3, 494.4)	332.1	(165.8, 665.2)	218.5	(54.6, 874.2)
IL-10 CM	304.3	(43.6, 2124.4)	0.4	(0.1, 2.2)	6.5	(0.1, 846.3)	686.6	(208.8, 2257.4)	180.6	(13.7, 2376.3)	55.0	(3.7, 819.0)
TNF-R2 CM	2359.1	(1379.8, 4033.3)	2312.6	(840.1, 6365.5)	2850.6	(2717.6, 2990.2)	2681.1	(1837.1, 3912.9)	3683.9	(2561.3, 5298.4)	4874.7	(3758.0, 6323.1)
TNF-R1 CM	531.1	(338.7, 832.9)	554.5	(499.2, 615.9)	638.7	(517.2, 788.9)	661.6	(513.1, 853.1)	1177.5	(780.4, 1776.5)	1208.5	(883.4, 1653.2)
TNFα/IL10 Homo	18.8	(10.4, 34.1)	66.5	(24.3, 181.9)	11.0	(5.5, 22.3)	13.9	(9.4, 20.5)	14.8	(8.1, 27.2)	15.3	(7.4, 31.9)
TNFα/IL10 CM	1.9	(0.4, 8.6)	105.2	(13.1, 844.8)	4.0	(0.1, 365.6)	0.8	(0.3, 2.5)	1.8	(0.2, 13.6)	4.0	(0.8, 20.2)

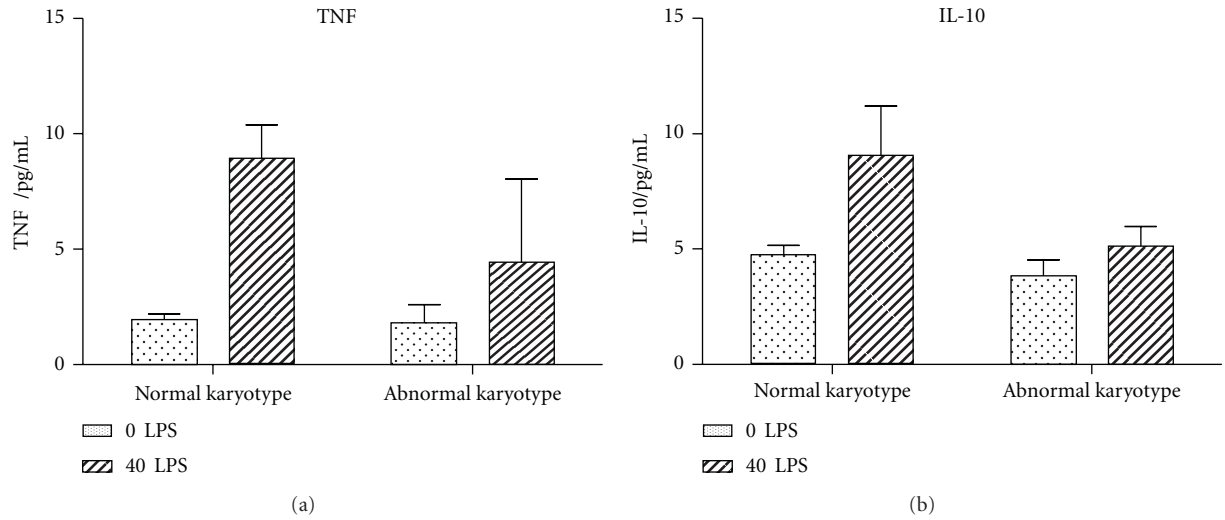

Figure 2: Levels of cytokines in monocytes ($n = 12$ with normal karyotype and $n = 14$ with abnormal karyotype). There was no significant difference in any of the intracellular levels of cytokines.

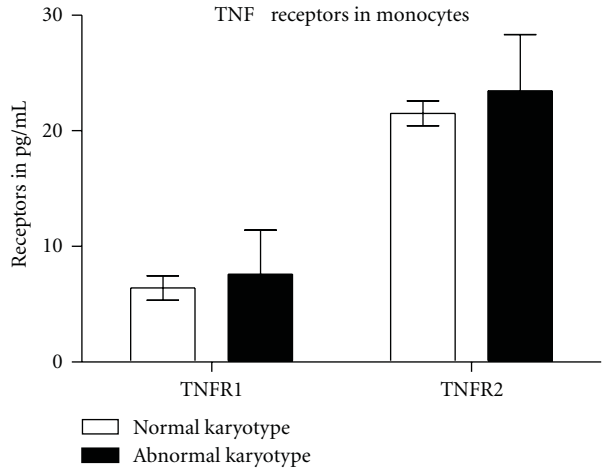

Figure 3: Levels of TNF-R1 and TNF-R2 in monocytes ($n = 12$ with normal karyotype and $n = 14$ with abnormal karyotype). There was no significant difference in any of the intracellular levels of receptors.

maternal systemic inflammatory response with an imbalance in the Th-1/Th-2 ratio in the maternal circulation. By contrast, in villous samples from the same groups, we found an increased secretion of inflammatory cytokine TNFα and its soluble receptors, TNF-R1 and TNF-R2, and the anti-inflammatory cytokine IL-10 in cases with an abnormal karyotype, compared to those presenting with a normal karyotype.

We have previously shown that in early pregnancy failure, the excessive entry of oxygenated maternal blood into the intervillous space has a direct mechanical effect on the villous tissue which becomes enmeshed inside large intervillous blood thrombi and an indirect O_2-mediated trophoblastic damage with increased apoptosis [21–23]. This phenomenon occurs at some point in miscarriages independently of the aetiology and in particular of the conceptus karyotype [24]. As all our villous tissue samples were obtained from missed miscarriage, the abnormal influx of maternal oxygenated blood would have had a similar impact on the villous tissue of both the cases presenting with and those presenting without a chromosomal karyotype at the time of the clinical diagnosis. This can explain why $O_2$and the number of days in culture had no impact on cytokine and receptor levels in our explant cultures experiments in both groups. The absence of significant difference in cytokine levels in the snap frozen samples (on day of collection) suggests that the difference seen in culture reflects functional changes.

In the present study, we also found that in early pregnancy (i.e., before 14 weeks), the amount of TNFα and IL-10 secreted *in vitro* by villous tissue from sporadic miscarriage was lower in the group with a normal karyotype. These data indicate that the karyotype of the conceptus has a direct impact on the secretion of cytokines by the villous tissue. An abnormal karyotype leads to an elevated local inflammatory response, confirmed by the significant rise in TNFα. This was accompanied by a rise in the anti-inflammatory cytokine IL-10 and the neutralizing soluble TNF-R1 and TNF-R2. An upregulation of the cytokine expression by the villous tissue has been reported in many cases of unexplained spontaneous miscarriages associated with a severe congenital infection presenting with maternal systemic symptoms [25]. In early miscarriages, there is massive destruction of maternal immunoglobulins in embryonic monocytes, with acute villitis in the placental barrier [26]. Some authors have therefore suggested that the upregulation of cytokines is deleterious to the developing placenta and fetus, probably as a consequence of vasoconstriction and/or direct cellular damage [27, 28]. Chromosomally abnormal spontaneous miscarriages may occur because of different mechanisms than chromosomally normal spontaneous miscarriages. Chromosome aberrations cause changes in placental morphology and function, including size, shape,

and vascularity and may affect rates of apoptosis of the stromal cells and cell proliferation in blood vessels during differentiation of chorionic villi [29].

In the maternal plasma, we observed inflammatory changes in the opposite direction than in villous tissue. The group presenting with a normal karyotype had a shift towards a Th-1 cytokine immune response in the circulation, reflected by a higher TNFα/IL-10 cytokine ratio than miscarriages with abnormal karyotype. Monocytes, in the normal karyotype group released a higher level of TNFα upon stimulation with LPS. This suggests that in the case of miscarriage of a karyotypically normal fetus, the maternal systemic inflammatory response is more sensitized. It is possible that in the case of karyotypically normal miscarriage, there is a systemic maternal immune response that causes the rejection of the fetus. In contrast, in cases with an abnormal karyotype, implantation would have already failed following the local excessive inflammatory reaction at the materno-fetal placental interface.

In recurrent miscarriage, the circulating cytokine levels and the decidual cytokine profile are different from those found in normal first trimester controls [30–33]. In particular, during periconception in recurrent miscarriage, higher circulatory levels of NK cells [34] and higher serum levels of macrophage migration inhibition factor [35] have been shown to be predictive of miscarriage of a conceptus with normal karyotype. In addition, women with recurrent miscarriages who miscarry a pregnancy with normal karyotype have decreased serum levels of TNFα [36] as early as 6-7 weeks of gestation. In our study, we have only looked at spontaneous sporadic miscarriages; however, miscarriages of normal karyotype are more likely to recur due to a possible underlying maternal immune problem [37].

The biological functions TNFα depends on its binding to two known receptors, TNF-R1 and TNF-R2 [38]. The soluble forms of these membrane receptors bind to TNFα with high affinity and can neutralize TNFα function [39, 40]. Yu et al., using flowcytometric and immunohistochemical measurement of TNF-R1 in the decidua, and detection of serum levels of soluble TNF-R1 by enzyme-linked immunoassays, found an association between the over-expression of TNF-R1 and early pregnancy failure [41]. In our study, villous secretion of soluble TNF-R1 in the culture medium and soluble TNF-R2 content in villous homogenate was significantly higher in cases presenting with abnormal karyotype.

Our data illustrate that the mechanisms leading to a miscarriage may depend on the karyotype of the conceptus. We suggest that there is a local functional disturbance in the karyotypically abnormal placental tissue while, in the case of a normal karyotype miscarriage, rejection occurs due to a maternal systemic inflammation.

Authors' Contribution

E. Jauniaux and S. Muttukrishna have equal contribution. J. Calleja-Agius collected the samples and performed the experiments and statistical analysis and wrote the first draft of the manuscript. S. Muttukrishna and E. Jauniaux contributed to the study design interpretation of the data and writing of the manuscript. S. Muttukrishna supervised the laboratory work and data analysis.

Ethical Approval

The procedures of this study received ethical approval from the the UCL Ethics Committee at The National Hospital for Neurology and Neurosurgery and Institute of Neurology Joint Research Ethics Committee. This research work is part of project entitled "Early Pregnancy Complications: What causes them and how can we improve diagnosis?" (Project ID: 07/QO512/41) and was ethically approved in April 2008.

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Research Article

Macrophage Migration Inhibitory Factor in Fetoplacental Tissues from Preeclamptic Pregnancies with or without Fetal Growth Restriction

Simona Cardaropoli,[1] Luana Paulesu,[2] Roberta Romagnoli,[2] Francesca Ietta,[2] Daniela Marzioni,[3] Mario Castellucci,[3] Alessandro Rolfo,[1] Elena Vasario,[1] Ettore Piccoli,[1] and Tullia Todros[1]

[1] *Department of Obstetrics and Gynecology, University of Turin, Sant'Anna Hospital, Via Ventimiglia 3, 10126 Torino, Italy*
[2] *Department of Physiology, University of Siena, Via Aldo Moro 4 53100 Siena, Italy*
[3] *Department of Molecular Pathology and Innovative Therapies, Marche Polytechnic University, Via Tronto 10/a 60020 Ancona, Italy*

Correspondence should be addressed to Simona Cardaropoli, simona.cardaropoli@unito.it

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The proinflammatory cytokine MIF (macrophage migration inhibitory factor) is involved in physiological and pathological processes in pregnancy. MIF maternal serum levels are increased in preeclampsia (PE). We hypothesize that pregnancy tissues are the source of MIF overexpression in PE. MIF protein was studied in maternal sera, placental tissues, fetal membranes, and umbilical cord of 8 control and 20 PE pregnancies: 10 with normal fetal growth (PE-AGA) and 10 with fetal growth restriction (PE-FGR). MIF levels were significantly higher in PE-AGA membranes than in controls and PE-FGR. In PE-FGR, MIF cord concentrations were higher than in PE-AGA while MIF placental levels were lower than in controls. MIF maternal serum levels were higher in PE, compared to controls, and the difference was mainly due to PE-FGR samples. These data support MIF involvement in PE pathogenesis and suggest that different pregnancy tissues contribute to MIF production in PE with and without fetoplacental compromise.

1. Introduction

Preeclampsia (PE) is the most serious syndrome of human pregnancy and it is potentially life-threatening for both mother and fetus. In developed countries, where the diagnosis and management of the disease is a major aim of prenatal care, maternal mortality attributable to PE has been reduced. However, perinatal and long-term morbidity and neurological sequelae, due to fetal growth restriction (FGR) and/or preterm delivery, are still critical problems [1, 2]. Nowadays, there are no effective interventions to prevent or cure PE except for a timed and often premature delivery [3]. This is partly due to the fact that the aetiology and the pathogenesis of the disease are still poorly understood.

It is widely accepted that a generalized endothelial dysfunction and an exaggerated inflammatory response are involved in the pathogenesis of PE [1, 2]. Furthermore, it is assumed that an inadequate trophoblast invasion and remodelling of maternal spiral arteries may cause or contribute to the pathogenesis of the disease [4]. These tissue-re-modelling processes are driven in part by placental cytokines.

Macrophage migration inhibitory factor (MIF) plays a pivotal role in inflammatory and immune diseases [5] and in inflammatory-like reproductive events as ovulation, menstrual cycle, and early placentation [6–8]. MIF was originally identified as a factor released by activated T-lymphocytes able to inhibit the random migration of macrophages *in vitro* [9]. Although macrophages and T-lymphocytes are the main sources of MIF, fibroblast, epithelial, and endothelial cells are also able to express and release MIF [10, 11]. MIF is also expressed in normal trophoblast [7] and membranes, and it is detectable in amniotic fluid and in maternal and fetal sera [12].

Traditionally, the major focus of MIF research has been on its role as a proinflammatory mediator. Indeed, it has been demonstrated that MIF directly or indirectly promotes the expression of a large panel of proinflammatory molecules, such as tumor necrosis factor (TNF)-α, interferon γ, interleukin- (IL-) 1β, IL-2, IL-6, IL-8, matrix metallo-proteinases (MMPs), nitric oxide, and products of the arachidonic acid pathway [5, 13].

We have previously reported that MIF maternal serum levels are increased in preeclamptic patients compared to normal pregnant women [14]. Based on these findings, we suggested that MIF might be involved in the pathogenesis of PE.

The aim of the present study was to verify the hypothesis that high MIF levels in preeclamptic maternal serum might derive from the fetoplacental unit. For this purpose we assessed the protein expression and localization of MIF in placental tissues, fetal membranes, and umbilical cords obtained from control and preeclamptic pregnancies.

2. Methods

We selected, classified, and managed pregnancies complicated by PE and controls whose placentae, fetal membranes and umbilical cords were processed and studied. The study was approved by our Piedmont Regional and Hospital Ethics Committee and informed consent was obtained from each woman.

Exclusion criteria were, multiple pregnancies, pregnancies complicated by prenatal or postnatal diagnosis of structural and/or chromosomal anomalies, and prepregnancy diseases (chronic hypertension, diabetes, etc.).

2.1. Study Population

2.1.1. Preeclamptic Cases. Twenty consecutive pregnancies complicated by PE were included in our study. Preeclampsia was defined by appearance of hypertension (systolic blood pressure $\geq$140 mmHg and/or diastolic blood pressure $\geq$90 mmHg) accompanied by proteinuria ($\geq$300 mg/24 h) after twenty weeks of gestational age in previously normotensive patients [15]. All patients had pathological uterine Doppler flow velocity waveforms (FVW), defined as a resistance index (RI) of >0.58 with or without the presence of bilateral notching [16].

Among these patients, two subgroups were identified based on the presence or absence of FGR: ten PE mothers delivered appropriate-for-gestational-age newborns (PE-AGA) and 10 PE pregnancies were complicated by FGR (PE-FGR).

The diagnosis of FGR was made according to the following criteria: ultrasound measurement of the fetal abdominal circumference below the 10th centile [17] or a growth velocity below the 10th percentile [18] and/or birth-weight below the 10th centile according to our birth-weight references [19], with abnormal FVW of the umbilical arteries [20]. All PE pregnancies were delivered by caesarean section.

2.1.2. Control Pregnancies. Controls were 8 normotensive pregnancies with normal fetal growth and normal uterine and umbilical Doppler FVWs, delivered at term by caesarean section, because of breech presentation or previous caesarean section.

In all cases and controls, pregnancies were dated by an ultrasound scan in the first trimester of pregnancy.

2.1.3. Clinical Parameters. The following data were collected for both cases and controls: maternal age at delivery, parity, smoking habits, body mass index (BMI = kg/m^2) at the beginning of pregnancy, gestational age at birth, gestational age at onset of PE, mode of delivery, neonatal sex and weight at birth (neonatal weight was also expressed as Z-score, that is, exact number of standard deviations from the mean for gestational age, using our birth-weight references [19]), placental weight, uterine and umbilical artery Doppler ultrasound velocimetry indexes, blood pressure, urinary protein levels, and exposure to drugs (such as antihypertensives, corticosteroids, antibiotics, aspirin).

2.2. Tissue Samples. Immediately after delivery, normal and pathological placentae (umbilical cord and membranes included) were transported from the delivery room to the laboratory and, after preliminary gross examination, two series of tissue samples were obtained: (a) three full-thickness samples of placental tissue were randomly collected from an intermediate zone between umbilical cord insertion and periphery, two samples of fetal membranes were taken far away from both the free edge and the placental plate, two umbilical cord samples were dissected from an intermediate zone between insertion and fetus; each sample was fixed in neutral buffered 10% formaldehyde for 24 hours and embedded in paraffin for immunohistochemistry; (b) further three samples of placental tissue, two of fetal membranes and two of cord tissue were collected as above described, put into cryovials and immediately frozen in liquid nitrogen then stored at -70°C until tissue lysate for MIF concentration analysis by a specific ELISA assay.

2.3. Blood Samples. Before delivery, peripheral venous blood samples were collected in vacutainer tubes (Becton Dickinson) without anticoagulant, from mothers with normal and pathological pregnancies. Serum was separated by centrifugation immediately after clotting and stored at -20°C until assayed. Concentration of MIF in maternal serum samples were determined by a MIF ELISA assay.

2.4. Tissue Lysate. Tissue lysates from placenta, membrane and cord samples, from normal and pathological (PE-AGA and PE-FGR) pregnancies, were obtained after complete homogenization in RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% (vol/vol) Triton X-100, 1% (wt/vol) Na deoxycholate, 0.1% (wt/vol) SDS, pH 7.5), and 3,000 g centrifugation at 4°C for 15 min. After total protein evaluation, tissue lysates were stored in aliquots at -70°C until assayed all together by MIF ELISA.

2.5. MIF ELISA. Concentration of MIF in tissue lysates as well as in maternal sera, from preeclamptic and control pregnancies were assayed by a colorimetric sandwich ELISA (enzyme-linked immunosorbent assay) as reported by Ietta et al. (2002) [12]. ELISA plates were coated with 100 μL of antihuman MIF monoclonal antibody (2.0 μg/mL) (R&D System) and incubated overnight at room temperature (RT). The plates were washed three times with Wash Buffer (10 mM PBS (pH 7.4), 0.05% (v/v) Tween 20), blocked by adding 300 μL blocking solution (10 mM PBS (pH 7.4), 1% (wt/v) bovine serum albumin (BSA), 5% (wt/v) sucrose, and 0.05% (wt/v) NaN_3), and incubated at RT for 1.5 h. After three washes, the samples and the standard, human recombinant MIF (R&D Systems), appropriately diluted in Tris-buffered saline-BSA (20 mM Tris-HCl, 150 mM NaCl (pH 7.3), 0.1% (wt/v) BSA, 0.05% (v/v) Tween 20), were added in duplicate (100 μL/w) and incubated for 2 h at RT. The plates were then washed three times and 100 μL of biotinylated goat antihuman MIF polyclonal antibody (200 ng/mL) (R&D Systems) was added to each well and incubated for 2 h at RT. The plates were washed again and streptavidin horseradish peroxidase (Zymed, San Francisco, Calif, USA) was added to each well and incubated for 20 min at RT. The plates were washed and 3,3′,5,5′-tetramethylbenzidine (Zymed) was added. After 30 min, the reaction was stopped by adding H_2SO_4 (0.1 M). The absorbance was measured at 450 nm using an ELISA SR 400 microplate reader (Sclavo, Siena, Italy). The MIF concentration was expressed as pg/mg of total tissue proteins, in tissues lysates, and as ng/mL, in serum samples. The sensitivity limit was 18 pg/mL. Intra- and interassay coefficients of variation (CV%) were 3.86 ± 0.95% and 9.14 ± 0.47%, respectively.

2.6. Immunohistochemistry. Paraffin sections from placenta, membrane and cord samples were analyzed for MIF expression by immunohistochemistry (IHC). From each sample, serial sections of 3 μm were obtained, mounted on 0.01% poly-lysine coated glass-slides, and air-dried for 24 h at 40°C.

IHC was performed using the Strept ABComplex/AP method. Sections were dewaxed, rehydrated, and washed in Tris-buffered saline [TBS; 20 mM Tris-HCl, 150 mM NaCl (pH 7.6)]. Antigen retrieval was carried out by incubating sections in sodium citrate buffer (10 mM, pH 6.0) in a microwave oven at 750 Watts for 5 min for three times and preincubated with normal rabbit serum to prevent nonspecific bindings. Slides were incubated overnight at 4°C with an anti-human MIF monoclonal antibody (R&D System Abingdon, UK), diluted 1 : 100 in TBS. Slides were washed and incubated with rabbit anti-mouse biotinylated antibody (DAKO, Copenhagen, Denmark) at a dilution 1 : 200 for 40 min. The alkaline phosphatase reaction was revealed by Sigma Fast (Sigma Aldrich, St. Louis, Mo, USA) as substrate. Sections were contrasted with Mayer's Hematoxylin, mounted, and examined under a light microscope. For each case, a negative control was obtained by using the antibody preadsorbed with the recombinant MIF at the concentration of 20 μg per mL of diluted antibody.

2.7. Statistical Analysis. Patient age, BMI, gestational age, neonatal and placental weight, birth weight Z-score, placental weight/neonatal weight ratio, blood pressure readings, and MIF concentrations were reported as mean and standard deviations (SDs). Means among groups were compared using a one-way analysis of variance (ANOVA). Tamhane post hoc tests, chosen to account for unequal variances, were calculated to identify significant differences between the dependent variables at $\alpha < 0.05$. Categorical and nominal values (parity, smoking habit, urinary protein levels, exposure to pharmaceuticals) were analyzed by the chi-squared test (χ^2). Fisher's exact test was used for small sample sizes. A value of $P \leq 0.05$ was considered significant. Statistical evaluation was performed using SPSS 18 for Windows (SPSS, Chicago, Ill, USA).

3. Results

3.1. Study Population. The three study groups, controls, PE-AGA, and PE-FGR, were comparable for maternal age, pre-pregnancy BMI and percentage of patients receiving antibiotics (Table 1).

All PE pregnancies differed from controls for gestational age at delivery, neonatal birth weight, and neonatal weight Z-score, blood pressure, urinary protein values and percentage of patients receiving corticosteroids or antihypertensives (Table 1). Smoking mothers were more frequent in PE (statistically significant in PE-FGR versus controls $P < 0.036$) than in controls and placental weight was significantly lower in PE with FGR ($P < 0.001$), while percentage of nulliparae was lower and placental weight/neonatal weight ratio was significantly higher in PE group with appropriate-for-gestational-age newborns (PE-AGA) compared to controls ($P = 0.041$ and $P = 0.018$, resp.) (Table 1).

The two subgroups of pregnancies complicated by PE did not significantly differ for percentage of nulliparae and smoking mothers, for gestational age at delivery, gestational age at onset of PE, blood pressure, and percentage of patients receiving drugs. In the PE-FGR group, neonatal and placental weight and neonatal weight Z-score were lower than in PE-AGA; moreover, there were differences between the two groups in urinary protein levels, umbilical and uterine artery Doppler ultrasound velocimetry indexes (Table 1).

3.2. MIF Concentration in Fetoplacental Tissues and Maternal Sera. Quantification of MIF protein by a specific ELISA assay revealed differences between pathological and normal control samples as described herein (Table 2, Figure 1).

MIF concentration was higher in placental tissues than in fetal membranes and umbilical cords in control pregnancies (Table 2). This scenario completely changed in the two subgroups of pregnancies complicated by PE. MIF concentration was higher in fetal membranes in PE-AGA and in umbilical cord in PE-FGR as compared to the other tissues. As for PE-AGA, the difference was significant between fetal membranes versus umbilical cord ($P = 0.005$) while in PE-FGR, it was significant between umbilical cord and fetal membranes

Table 1: Clinical characteristics of control and pathological pregnancies.

	Controls	PE-AGA	PE-FGR	*P* value [a]
Number of patients	8	10	10	
Maternal age at delivery (years), mean (SD)	32.8 (4.3)	34.5 (3.8)	32.5 (5.3)	n.s.
Nulliparae, number and %	7* (87.5)	5* (50)	8 (80)	*0.041
Smoking mothers, number and %	0° (0)	2 (20)	5° (50)	°0.036
Prepregnancy BMI (Kg/m^2), mean (SD)	21.4 (2.5)	24.2 (6.1)	24.4 (4.7)	n.s.
Gestational age at delivery (weeks), mean (SD)	37.5*° (3.2)	30.6* (2.6)	30.0° (2.5)	*° <0.001
Neonatal birth weight (g), mean (SD)	3254*° (439)	1360*^ (309)	1001°^ (277)	*° <0.001; ^0.048
Neonatal weight Z-score, mean (SD)	0.26*° (0.95)	−1.02*^ (0.30)	−2.02°^ (0.58)	*0.018; ° <0.001; ^0.004
Placental weight (g), mean (SD)	541° (81)	428^ (203)	206°^ (58)	° <0.001; ^0.021
Placental weight/neonatal weight ratio, mean (SD)	0.17* (0.01)	0.31* (0.13)	0.23 (0.10)	*0.018
Gestational age at onset of PE (weeks), mean (SD)	n.a.	29.3 (3.1)	28.5 (3.1)	n.s.
Blood pressure (mmHg), mean (SD):				
Systolic	110.9*° (9.4)	158.4* (6.6)	150.0° (18.9)	*° <0.001
Diastolic	71.8*° (7.5)	96.4* (9.5)	93.9° (9.8)	*° <0.001
Proteinuria, number and %:	*°	*^	°^	
<1 g/24 h	0 (0)	0 (0)	5 (50)	
<5 g/24 h	0 (0)	7 (70)	3 (30)	*° <0.001; ^0.033
≥5 g/24 h	0 (0)	3 (30)	2 (20)	
Umbilical arterial mean pulsatility index, mean (SD)	n.a.	1.10 ^ (0.17)	2.03 ^ (0.32)	^ <0.001
Uterine arterial mean resistance index, mean (SD)	n.a.	0.67 ^ (0.07)	0.76 ^ (0.05)	^0.021
Patients receiving, number and %:				
Corticosteroids	1*° (12.5)	9* (90)	10° (100)	* <0.001; °0.003
Antihypertensives	0*° (0)	10* (100)	10° (100)	*° <0.001
Antibiotics	2 (25.0)	2 (20)	0 (0)	n.s.

PE: preeclampsia.
PE AGA: preeclamptic pregnancies with appropriate-for-gestational-age newborns.
PE-FGR: preeclamptic pregnancies with fetal growth restriction.
BMI: body mass index.
n.s.: not significant.
n.a.: not available.
[a]*P* values were calculated by ANOVA test, followed by Tamhane test for pairwise comparison, or by chi-squared test (χ^2).
*Comparison between Controls and PE-AGA group.
°Comparison between Controls and PE-FGR group.
^Comparison between PE-AGA and PE-FGR groups.

Table 2: Concentration of MIF in normal and pathological samples.

	Number of patients	Placental tissue (pg/mg)	Fetal membranes (pg/mg)	Umbilical cord tissue (pg/mg)	*P* value[a]
Controls	8	163,8* (112,4)	87,7 (88,1)	72,3* (46,4)	*0,034
All PE	20	119,5 (80,3)	112,8 (105,5)	85,6 (58,2)	n.s.
PE-AGA	10	142,8* (94,6)	162,2° (116,7)	60,9*° (48,4)	*0,006; °0,005
PE-FGR	10	93,7 (51,9)	58,0° (54,5)	107,5° (58,5)	°0,038

PE: preeclampsia.
PE-AGA: preeclamptic pregnancies with appropriate-for-gestational-age newborns.
PE-FGR: preeclamptic pregnancies with fetal growth restriction.
n.s.: not significant.
[a]*P* values were calculated by ANOVA test, followed by Tamhane test for pair-wise comparison.
*Comparison between placental and umbilical cord MIF concentrations.
°Comparison between fetal membranes and umbilical cord MIF concentrations.

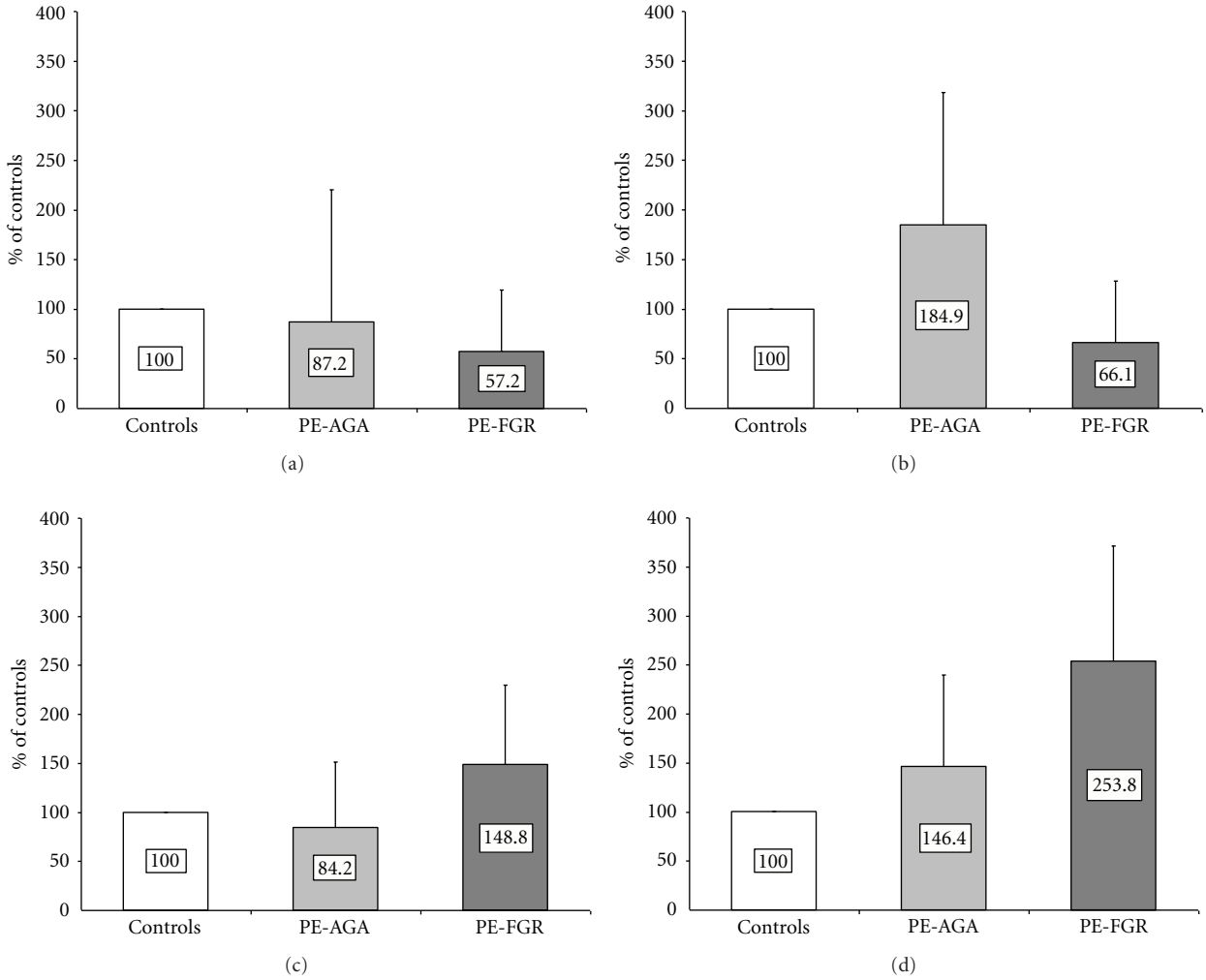

Figure 1: MIF ELISA assay in normal and pathological samples. (a) placenta, $^{*}P < 0.001$ (control tissues versus PE-FGR); (b) fetal membranes, $^{*}P = 0.03$ (control tissues versus PE-AGA) and $^{**}P = 0.004$ (PE-AGA versus PE-FGR); (c) umbilical cord, $^{*}P = 0.047$ (PE-AGA versus PE-FGR); (d) maternal serum,$^{*}P = 0.023$ (CTRL versus PE-FGR). P values were calculated by ANOVA test, followed by Tamhane test for pair-wise comparison.

($P = 0.038$) (Table 2). No significant differences were found in tissues when PE pregnancies were considered all together (with or without FGR) (Table 2).

Figure 1 shows comparison in MIF concentration between pathological and normal control pregnancies in feto-placental tissues and maternal serum samples. PE values are reported as percentage of controls. MIF levels in placental tissues were significantly lower in PE pregnancies with FGR compared to controls ($P < 0.001$) (Figure 1(a)). In fetal membranes, MIF concentration was significantly higher in PE-AGA compared to normal membranes and PE-FGR samples ($P = 0.03$ and $P = 0.004$, resp.) (Figure 1(b)), while PE-FGR umbilical cord tissues had higher MIF concentration compared to levels measured in PE-AGA ($P = 0.047$) and controls (not significant) (Figure 1(c)). Finally, the increase of MIF levels in PE maternal sera was mainly due to PE-FGR samples, in fact MIF concentration in PE-FGR sera resulted significantly higher compared to control samples ($P = 0.023$) (Figure 1(d)). No significant difference was observed for any tissue when PE pregnancies were considered all together except for maternal serum: MIF maternal serum levels in PE pregnancies were significantly higher (5126 ± 2902 ng/mL) than in controls (2467 ± 703 ng/mL, $P = 0.020$).

3.3. MIF Immunoreactivity in Normal and Pathological Tissues. Localisation of MIF by immunohistochemistry showed MIF protein in the villous trophoblast and fetal endothelial cells both in controls and PE placental tissues (Figures 2(a), 2(b), and 2(c)). Differences between control and pathological tissues were due to the appearance of a MIF immunoreactivity in PE-FGR intervillous space, mainly at the external border of villi (Figures 2(a) and 2(c)).

All cell types of fetal membranes were positive for MIF (Figures 2(d), 2(e), and 2(f)). PE-AGA fetal membranes showed a stronger MIF immunostaining of epithelial cells of amnion side and decidual cells (Figure 2(e)) compared to controls (Figure 2(d)) and PE-FGR (Figure 2(f)).

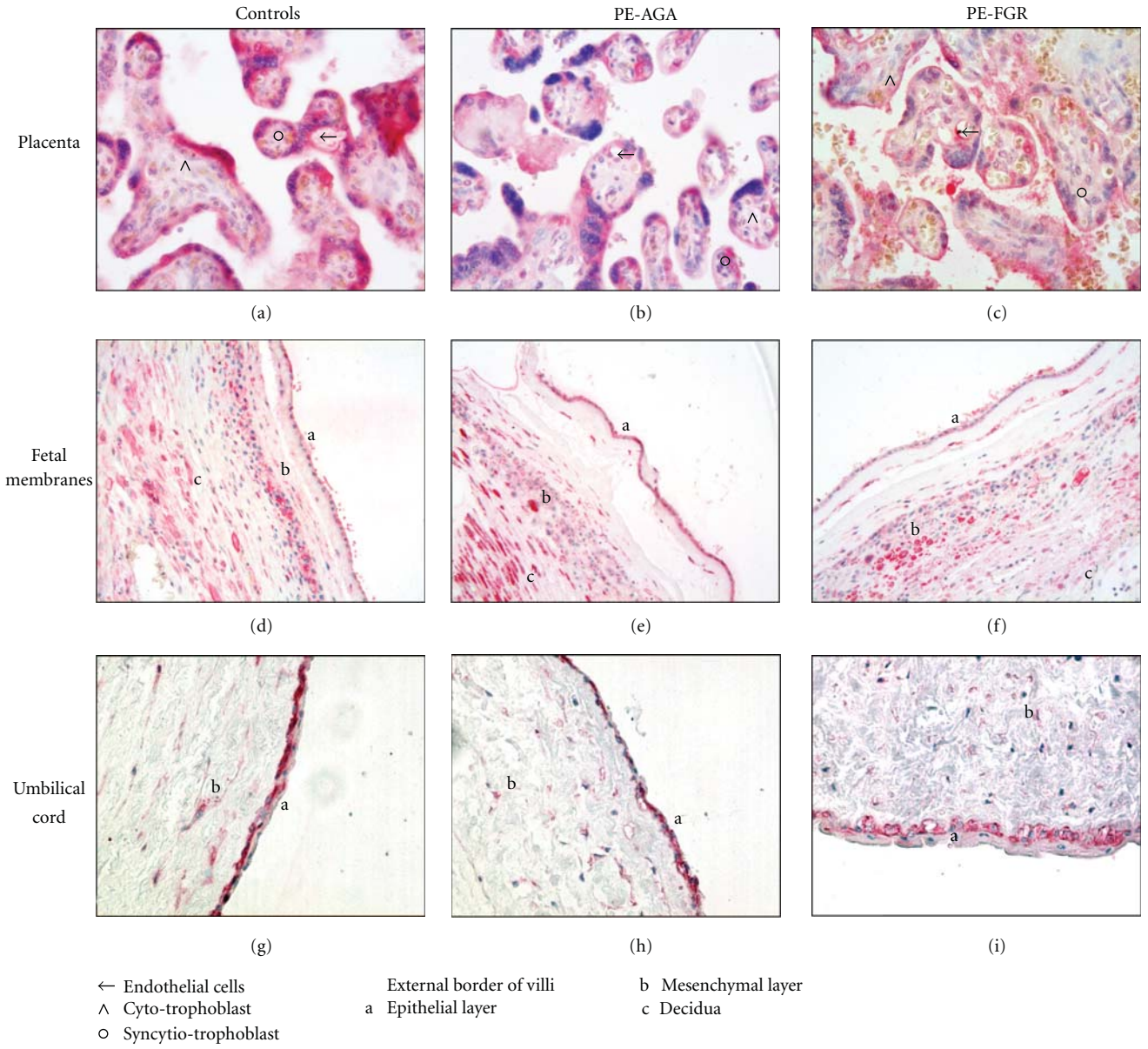

Figure 2: MIF immunoreactivity in tissues from normal and pathological pregnancies. Reddish color indicates positive staining for MIF. Representative images for placenta, fetal membranes, and umbilical cord from normal (a, d, g), PE-AGA (b, e, h) and PE-FGR (c, f, i) pregnancies, respectively. (←): endothelial cells; (^): cytotrophoblast; (°): syncytiotrophoblast; (*): external border of villi; a: epithelial layer; b: mesenchymal layer; c: decidua. Original magnification: 40x for placenta panel, 20x for fetal membranes and umbilical cord panels.

Immunohistochemistry of umbilical cord tissue sections revealed MIF expression in all cell types, and immunostaining resulted stronger in external epithelial cell layer. No distinguishable differences were observed between normal and pathological cord tissues (Figures 2(g), 2(h), and 2(i)).

4. Discussion

In the present study we confirmed our previous findings [14] showing that MIF maternal serum levels are higher in PE patients compared to normal pregnant women. Moreover, we investigated MIF protein concentration and localization in the placenta, fetal membranes, and umbilical cord in order to clarify the possible role of fetoplacental tissues in determining higher MIF serum levels in PE patients. Our data on MIF immunoreactivity in normal term placentas and fetal membranes confirm previously published works [12, 21], whereas our observations in the umbilical cord support *in vitro* studies conducted on human umbilical vein endothelial cells [22]. Herein we show for the first time to our knowledge that MIF is expressed in both control and PE fetoplacental tissues.

In this paper, we did not find differences in MIF concentration in tissues obtained from normal and PE pregnancies, when PE pregnancies were considered all together (with or without FGR). Nevertheless, when PE pregnancies with normal fetal growth and fetoplacental hemodynamics and PE complicated by FGR (with abnormal umbilical artery Doppler FVW) were considered separately, the picture completely changed. Placental MIF concentration was significantly lower in PE-FGR but not in PE-AGA compared to controls. MIF expression in fetal membranes was significantly higher in PE-AGA compared to both PE-FGR and controls, while MIF protein was significantly over-expressed in PE-FGR cord tissue compared to PE-AGA and higher compared to controls.

Noteworthy, the relative concentrations of MIF in placenta, fetal membranes, and umbilical cord were different among groups: they were higher in the placenta of controls, in the fetal membranes of PE-AGA, and in umbilical cord of PE-FGR. As above reported for comparison between normal and pathological pregnancies, differences were not statistically significant when PE pregnancies were considered all together. The significant difference in MIF maternal serum levels is mainly due to PE-FGR cases.

As shown in our previous study on PE, the increased serum MIF levels in PE-FGR cases were not due to β-methasone administration because no significant differences were detected between before and after corticosteroids treatment [14]. These findings are consistent with results by Isidori et al., (2002), showing that the response of serum cortisol to stimulation of the hypothalamo-pituitary-adrenal axis was not associated with a corresponding rise in plasma MIF [23].

It is well known that PE is a syndrome where similar symptoms could origin from different etiopathological pathways. In 2005, Redman and Sargent [1] introduced the concept of two different PE diseases: placental and maternal. Placental PE is characterized by an hypoxic placenta subjected to oxidative stress, while maternal PE arises from the interaction between a normal placenta and a maternal system susceptible or suffering of microvascular diseases, as well as long-term hypertension and/or diabetes [1]. Since FGR with abnormal umbilical Doppler FVW indicates placental compromise, we used fetal growth (FGR versus AGA) as a proxy for the definition of "placental" versus "maternal" PE. We have recently demonstrated that the enzyme HtrA1, involved in the physiological development of many organs, is differentially regulated in PE-AGA and PE-FGR placentas [24] as well as the transcription factors JunD and c-jun, implicated in regulating cytotrophoblast proliferation and differentiation, showing an opposite modulation in PE-AGA and PE-FGR [25]. Moreover, Ornaghi and colleagues observed that placental expression of anticoagulant protein Annexin 5 was significantly lower only in PE complicated by FGR—but not in PE-AGA—compared to controls [26]. Often in the literature "placental PE" and "maternal PE" are used as synonymous of "early onset" or "severe" PE and "late onset" or "mild" PE, respectively. Our study population shows that this not always true; in fact, gestational age at onset and at delivery was the same in both PE-AGA and PE-FGR groups. Moreover, severe PE complications, such as HELLP syndrome, were present in both groups.

In the light of the above observations, our findings can explain the differential role of fetoplacental compartments for increased MIF maternal serum levels in PE.

4.1. PE-FGR. Compared to controls, these cases were characterized by a significantly lower mean MIF protein content in the placenta. There were no difference in localization (mainly in the external layer of chorionic villi, the syncytiotrophoblast), except for the presence of MIF immunoreactivity in the intervillous space in pathological placentas.

The lower placental protein content could be the consequence of a defective translation or increased protein degradation, since MIF mRNA expression was not reduced (data not shown) among groups. An alternative explanation could be an increased MIF release in the intervillous space, thus explaining the high maternal serum levels observed in this subgroup of patients. A comparable phenomenon was observed in the epithelium of bovine epididymis and in urothelial cells of human bladder [27, 28]. In animal models of bladder inflammation and injury, it was shown that MIF protein amounts are decreased in rat urothelium and increased in the bladder lumen [28, 29]. Moreover, it was demonstrated that influenza A virus infection induces a decline of intracellular MIF protein in normal human bronchial epithelial cells, while extracellular MIF levels increases [30]. Further studies are required to demonstrate *in vitro* MIF release from PE and normal placental tissues.

The higher MIF levels that we found in PE-FGR umbilical cord were mainly localised in the epithelial and stromal cells of Wharton's jelly. FGR is due to abnormal development of the villous tree, which in turn impairs feto-maternal nutrient and gas exchanges, inducing fetal hypoxia. Since hypoxia induces MIF production [31], the low-oxygenated environment typical of PE-FGR placentas could be the most likely candidate for the increased MIF levels observed in the umbilical cord.

4.2. PE-AGA. This population was characterized by a significantly higher MIF expression in fetal membranes compared to controls. A similar increase of MIF immunostaining was found in fetal membranes of *Plasmodium falciparum* infected placentae [32]. Epithelial layer of membranes was previously studied as the source of MIF in amniotic fluid [12, 33] and MIF levels in amniotic fluid were increased in inflammatory conditions [34, 35]. It is plausible that fetal membranes could be the source of the slight increase in MIF maternal serum levels observed in PE-AGA.

In conclusion, our study further supports the evidence that MIF-related inflammation plays a pivotal role in the pathogenesis of PE. Our data provided new insights on the tissues responsible for the increased maternal MIF serum levels in PE, although it does not answer the question whether increased maternal MIF serum levels are the cause or the consequence of inflammation. Indeed, MIF was shown to upregulate and to be upregulated by proinflammatory stimuli [5, 36]. Of clinical relevance, we were able to

discriminate between placental and maternal preeclampsia on the base of MIF source within fetoplacental tissues.

Conflict of Interests

The authors reported no potential conflict of interest.

Acknowledgments

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Clinical Study

Follicular Proinflammatory Cytokines and Chemokines as Markers of IVF Success

Aili Sarapik,[1] Agne Velthut,[2,3] Kadri Haller-Kikkatalo,[1,3,4,5] Gilbert C. Faure,[6] Marie-Christine Béné,[6] Marcelo de Carvalho Bittencourt,[6] Frédéric Massin,[6] Raivo Uibo,[1,3] and Andres Salumets[3,4]

[1] *Department of Immunology, Institute of General and Molecular Pathology, University of Tartu, Ravila 19, Tartu 50411, Estonia*
[2] *Institute of Gene Technology, Tallinn University of Technology, Akadeemia Street 15, Tallinn 12618, Estonia*
[3] *Competence Centre on Reproductive Medicine and Biology, Tiigi 61b, Tartu 50410, Estonia*
[4] *Department of Obstetrics and Gynecology, University of Tartu, L. Puusepa 8, 51014 Tartu, Estonia*
[5] *Women's Clinic, Tartu University Hospital, L. Puusepa Street 1a, 50406 Tartu, Estonia*
[6] *Laboratoire d'Immunologie, Université Henri Poincaré, BP 184, Vandoeuvre lès Nancy, 54500 Nancy, France*

Correspondence should be addressed to Kadri Haller-Kikkatalo, kadri.haller-kikkatalo@ut.ee

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Cytokines are key modulators of the immune system and also contribute to regulation of the ovarian cycle. In this study, Bender MedSystems FlowCytomix technology was used to analyze follicular cytokines (proinflammatory: IL-1β, IL-6, IL-18, IFN-γ, IFN-α, TNF-α, IL-12, and IL-23;, and anti-inflammatory: G-CSF), chemokines (MIP-1α, MIP-1β, MCP-1, RANTES, and IL-8), and other biomarkers (sAPO-1/Fas, CD44(v6)) in 153 women undergoing *in vitro* fertilization (IVF). Cytokine origin was studied by mRNA analysis of granulosa cells. Higher follicular MIP-1α and CD44(v6) were found to correlate with polycystic ovary syndrome, IL-23, INF-γ, and TNF-α with endometriosis, higher CD44(v6) but lower IL-β and INF-α correlated with tubal factor infertility, and lower levels of IL-18 and CD44(v6) characterized unexplained infertility. IL-12 positively correlated with oocyte fertilization and embryo development, while increased IL-18, IL-8, and MIP-1β were associated with successful IVF-induced pregnancy.

1. Introduction

Immunological abnormalities have been implicated in female reproductive failure, but whether these represent a cause or effect is unknown [1, 2]. According to our previous research, cellular and, particularly, humoral autoimmunologic perturbations are responsible for development of female infertility. Disturbances in the humoral immune system may lead to impairments in ovarian folliculogenesis [3–5], a long and complex process in which both the endocrine and immune systems play significant roles.

Cytokines, originally identified as products of immune cells, are important mediators of immune responses. These proteins are able to stimulate or inhibit cell growth, regulate cell differentiation, induce cell chemotaxis, and modulate the expression of other cytokines. However, recent research has indicated that cytokines are synthesized by a broad range of nonimmune cell types, including the normal ovarian cells. Cytokine function in the ovary has been described as promoting processes of follicular growth, steroidogenesis, recruitment and activation of leukocytes necessary for ovulation and tissue remodelling during ovulation, luteinization, and luteolysis [6].

To gain a more detailed understanding of the cytokines involved in female fertility and their role in pregnancy outcome, we assessed 16 different follicular cytokines during infertility treatment. In particular, we evaluated the cytokines for Th1/proinflammation (interleukin- (IL-) 1β, IL-6, IL-12, IL-18, IL-23, interferon (IFN)-γ, IFN-α, and tumor necrosis factor-(TNF-) α) and anti-inflammation (granulocyte colony stimulating factor (G-CSF)), the principal chemokines (macrophage inflammatory protein- (MIP-) 1α, MIP-1β, monocyte chemotactic protein- (MCP-) 1, regulated on activation, normal T expressed and secreted

(RANTES) and IL-8), and other biomarkers (soluble apoptosis antigen (sAPO)-1/Fas and CD44 variant isoform CD44(v6)) secreted into the follicular fluid. The cytokines chosen for evaluation were shown in our previous study to be appreciably expressed in follicular granulosa cells at the mRNA level [7]; moreover, the importance of these particular cytokines in ovarian function has been proposed by others [8].

IL-1β, IL-6, and IL-18 are key mediators of inflammation and mediate many pathways of the normal immune response [9–11]. Human IFN-α comprises a family of extracellular signalling proteins with demonstrated antiviral, antiproliferating, and immunomodulatory activities [12]. The type II interferon IFN-γ is another proinflammatory cytokine and has been implicated in the development of a variety of autoimmune diseases [13]. IL-12 regulates cell-mediated immune responses. The p40 subunit of IL-12 is shared with IL-23 and is essential for recruitment and activation of many inflammatory cell types. Both of these cytokines interact with the innate and adaptive immune systems [14]. TNF-α, an acute phase protein, is critically involved in innate immune responses caused by pathogen exposure [15] but can also mediate noninfectious inflammatory processes such as autoimmunity and cancer [16].

Among the chemokines examined in this study, IL-8 is a neutrophil-specific factor involved in inflammatory processes and angiogenesis [17]. MIP-1α and MIP-1β are known as CC chemokines, and both act as chemoattractants for T cells and monocytes to mediate beneficial inflammatory processes, such as wound healing [18]. Meanwhile, MCP-1 and RANTES are potent chemoattractants of monocytes and T lymphocytes [19].

The other biomarkers examined in this study are established immunomodulators. G-CSF acts as a growth factor for haematopoietic cells [20]. APO-1 regulates tissue homeostasis by acting as the receptor for Fas ligand, the binding of which triggers a signaling cascade that leads to apoptosis inhibition [21]. And CD44(v6), a splice variant of the CD44, is a transmembrane glycoprotein associated with cell adhesion and has mostly been investigated in tumours [22].

In recent decades, above-mentioned cytokines have become the subject of studies examining normal mammalian reproduction [23], which have indicated a significant role for these factors in supporting female fertility. Thus, we carried out a simultaneous (multiplex) examination of these cytokines and biomarkers in follicular fluid of infertile women in order to assess their effects on oocyte and embryo quality and on pregnancy outcome of *in vitro* fertilization (IVF) treatment. The approach of cytokine profiling using multiplex assays offers a promising tool for identifying condition-specific biomarker panels with high accuracy [24]. We employed the Bender MedSystems FlowCytomix platform, which uses antibody-coated autofluorescent beads to simultaneously measure corresponding analytes from small sample volumes and low concentrations [24], facilitating time- and cost-efficient high-throughput screening. In addition, we sought to determine the origin of the secreted cytokines by performing mRNA analysis from two distinct follicular somatic cell populations: mural and cumulus granulosa cells (MGC and CGC, resp.).

2. Materials and Methods

2.1. Patients. The Ethics Committee on Human Research of the University of Tartu approved this study, and informed consent was obtained from all patients. The study group consisted of 153 women, aged 33.3 ± 4.5 years (mean ± standard deviation), who underwent IVF at Nova Vita Clinic between 2007 and 2010. IVF with intracytoplasmic sperm injection (ICSI) was performed in all women in this cohort, and the case was either male factor infertility or previous oocyte fertilization failure. The causes of infertility were distributed as follows: male factor infertility (43.8%, $n = 67$), tubal factor infertility (TFI; 28.8%, $n = 44$), polycystic ovary syndrome (PCOS; 5.2%, $n = 8$), endometriosis (15.0%, $n = 23$), unexplained infertility (4.6%, $n = 7$), and other reasons (2.6%, $n = 4$).

Ovarian hormonal stimulation was conducted according to a protocol of gonadotrophin-releasing hormone (GnRH) antagonist (Cetrotide; Merck Serono, Geneva, Switzerland) administered with recombinant follicle-stimulating hormone (Gonal-F; Merck Serono or Puregon, Schering-Plough, Kenilworth, NJ, USA). ICSI was performed at 4–6 h after oocyte pickup (OPU), resulting in a 68.9% fertilization rate. ICSI-derived embryos were cultured up to 48 h, after which good-quality embryos were identified by the presence of at least four blastomeres and ≤20% fragmentation. The rate of good-quality embryos was calculated as the proportion (%) of good-quality embryos out of all fertilized oocytes. Two embryos were chosen for transfer to the uterus, and 25.5% of clinical pregnancies resulted per embryo transfer. Clinical confirmation of intrauterine pregnancy was made using an ultrasound scan performed at the 6th or 7th week after transference. Follicular fluid samples from each individual were taken from a single follicle on the day of OPU and stored at −80°C until further use.

2.2. Flow Cytometry Analysis. Altogether, 16 biomarkers (divided into two 8 plexes) were evaluated from the individual follicular fluid samples by using a commercially available FlowCytomix Human Basic Kit Assay (Bender MedSystems, Vienna, Austria) and following the manufacturer's instructions. Quantitation measurements were performed by flow cytometer instrument FC 500 and accompanying CXP Software (Beckman Coulter, Calif, USA). The first 8 plex consisted of: IL-23, sAPO-1/Fas, MIP-1β, MIP-1α, CD44(v6), IL-8, G-CSF, and RANTES. The second 8-plex consisted of IL-12p70, IFN-γ, MCP-1, IL-6, IFN-α, IL-18, Il-1β, and TNF-α. Samples were prepared for processing by first thawing follicular fluids and centrifuging the whole volume at 450 g for 10 min. The resulting supernatants were used for analysis. FlowCytomix Pro 2.3 Software was used to perform calculations (Bender MedSystems). Standard curves for each biomarker were generated with manufacturer-supplied reference analyte (pg/mL concentrations). The concentration of a biomarker was calculated as

Table 1: List of primers used for real-time PCR analysis.

Gene	Forward primer	Reverse primer	NCBI reference
G-CSF [25]	GCTTGAGCCAACTCCATAGC	CAGATGGTGGTGGCAAAGTC	NM_001178147.1, NM_172220.2, NM_172219.2, NM_000759.3
IL-23A [26]	TGTTCCCCATATCCAGTG	TCCTTTGCAAGCAGAACTGA	NM_016584.2
IFN-γ	TGATGGCTGAACTGTCGCCAGC	CTGGGATGCTCTTCGACCTCGA	NM_000619.2
MIP-1α	TCAGAAGGACACGGGCAGCAGA	TCAGCAGCAAGTGATGCAGAGAAC	NM_002983.2
sAPO-1/Fas	CCAAGTGCAAAGAGGAAGTGAAGAG	TGGTTTTCCTTTCTGTGCTTTCTGC	NM_152871.2
CD44(v6)	GCTACCACAGCCTCAGCTCA	ACCTCGTCCCATGGGGTGTGA	NA*

*The forward primer was designed to cross the junction between exons 5 and 11, characteristic for only CD44(v6)-soluble splice isoform not described in NCBI database.

mean fluorescent intensity divided by single median standard curve.

2.3. Gene Expression Analysis. Gene expression studies of the measured cytokines were performed by real-time PCR of mRNA isolated from MGC and CGC from six patients. MGC were obtained from follicular fluid after OPU, and CGC were collected 4 h after OPU during oocyte denudation with bovine type IV-S hyaluronidase (Sigma-Aldrich, St-Louis, Mo, USA). The detailed isolation protocol has been previously published [7]. For leukocyte elimination, the MGC pool was incubated with CD45-coated magnetic beads (Dynabeads; Invitrogen, Oslo, Norway) for an additional 1 h at 4°C, followed by magnet-based cell sorting (DynaMag-15; Invitrogen) according to the manufacturer's protocol. Total RNA was extracted, and real-time PCR analysis was performed using either commercially available real-time PCR arrays (products PAHS-011A and PAHS-021A; SABiosciences, Frederick, Md, USA) or in-house designed and synthesized primers when the desired transcripts were not included in the kits or the quality of amplification, and melting curves were not satisfactory. Primers for Fas were designed to exclusively detect the soluble isoform, and those for CD44 were designed to amplify only exon 11 (the variable region 6). All primer sequences used in this study are listed in Table 1.

For double-stranded cDNA synthesis, 1 μg of high-quality total RNA was treated with DNase (Fermentas, Burlington, ON, Canada) and reverse transcribed to cDNA using the RT^2 First Strand Kit (SABiosciences) according to the manufacturer's protocols. RT^2 SYBR Green/ROX qPCR Master Mix (SABiosciences) and cDNA template were added to the array and product amplification was performed on a 7500 real time PCR System (Applied Biosystems, Foster City, Calif, USA). Those real-time PCR reactions using in-house primers were performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7900HT real-time PCR instrument (Applied Biosystems).

Results were analyzed with instrument-specific software using the $\Delta\Delta$Ct relative quantification method. Three housekeeping genes were used for normalization of the amplification data: beta actin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein RPL13A.

2.4. Statistical Analysis. The R2.3.1 A Language and Environment (Free Software Foundation, Boston, Mass, USA) was used to perform *t*-, Mann-Whitney *U*- and proportion tests and adjusted simple regression analysis. A P value <0.05 was considered as indicative of statistical significance.

3. Results

Table 2 summarizes the clinical data and infertility treatment parameters, while Table 3 lists detected concentrations of the tested biomarkers. Figure 1 summarizes the main associations observed between the clinical data and the levels of follicular biomarkers, according to analysis by adjusted regression models.

3.1. Associations between Infertility Cause and Levels of Biomarkers in Follicular Fluid. Patients characterized by male factor infertility represented the reference group in simple regression analysis, unless otherwise stated. Our results indicated that women with TFI had lower concentrations of IL-1β-adjusted $r = -12.6$ pg/mL, $P = 0.037$), and lower IFN-α levels were also significantly associated with TFI when the status of current smoking was included in the model (adjusted $r = -13.9$ pg/mL, $P = 0.046$).

PCOS patients were characterized by significantly higher follicular levels of CD44(v6) (age-adjusted $r = 2072.7$ pg/mL, $P = 0.010$) and MIP-1α (adjusted for age, cause of infertility, and follicular count in prestimulatory ovary $r = 3111.7$ pg/mL, $P = 0.007$). Women with endometriosis presented with higher levels of IL-23 than male factor infertility patients (adjusted by follicular number prior to stimulation $r = 157.1$ pg/mL, $P = 0.025$). Moreover, when compared to TFI patients, women with endometriosis had higher levels of follicular TNF-α (age-adjusted $r = 2.6$ pg/mL, $P = 0.047$) and IFN-γ (adjusted by follicular number prior to stimulation $r = 16.4$ pg/mL, $P = 0.030$). In women with unexplained infertility, significantly lower levels of follicular CD44(v6) were measured, as compared to male factor infertility patients (age-adjusted $r = -1888.4$ pg/mL, $P = 0.025$). However, when compared to TFI patients, the unexplained infertility patients also had lower levels of IL-18 (age-adjusted $r = -186.7$ pg/mL, $P = 0.021$).

Table 2: Clinical and IVF treatment parameters of patient groups.

	Male factor infertility ($N = 67$)[a]	Tubal factor infertility ($N = 44$)	Polycystic Ovary Syndrome ($N = 8$)	Endometriosis ($N = 23$)	Unexplained infertility ($N = 7$)	Other reasons ($N = 4$)	Total ($N = 153$)
Health parameters							
Age (years[†])	32.6 ± 4.3	34.7 ± 5.0[b]	34.8 ± 3.2	31.8 ± 3.9	32.7 ± 2.9	36.0 ± 5.9	33.3 ± 4.5
Infertility[‡]							
Primary	42 (62.7%, 50.0–73.9)	15 (34.1%, 20.9–50.0)[c]	6 (75.0%, 35.6–95.5)	18 (78.3%, 55.8–91.7)	6 (85.7%, 42.0–99.2)[c]	2 (50.0%, 15.0–85.0)	89 (58.2%, 49.9–66.0)
Secondary	25 (37.3%, 26.1–50.0)	29 (65.9%, 50.0–79.1)[c]	2 (25.0%, 4.5–64.4)	5 (21.7%, 8.3–44.2)	1 (14.3%, 0.8–58.0)	2 (50.0%, 15.0–85.0)	64 (41.9%, 34.0–50.0)
Parity (N[†])	0.3 ± 0.5	0.5 ± 0.7[b]	0.3 ± 0.5	0.1 ± 0.3	0.1 ± 0.4	0.5 ± 0.6	0.3 ± 0.6
S-FSH (U/L[†])	7.6 ± 2.3	8.2 ± 3.2	7.0 ± 1.9	7.5 ± 2.7	6.9 ± 0.3	6.5 ± 2.2	7.7 ± 2.6
Smoking[‡]							
Never smoker	52 (78.8%, 66.7–87.5)	32 (76.2%, 60.2–87.4)	6 (75.0%, 35.6–95.5)	20 (87.0%, 65.3–96.6)	5 (71.4%, 30.3–94.9)	2 (66.7%, 12.5–98.2)	117 (77.5%, 69.8–83.7)
Past smoker	7 (10.6%, 4.7–21.2)	4 (9.5%, 3.1–23.51)	1 (12.5%, 0.7–53.3)	1 (4.3%, 0.2–24.0)[c]	1 (14.3%, 0.8–58.0)	1 (33.3%, 1.8–87.5)[c]	15 (9.9%, 5.9–16.1)
Current smoker	7 (10.6%, 4.7–21.2)	8 (19.0%, 9.1–34.6)	1 (12.5%, 0.7–53.3)	2 (8.7%, 1.5–29.5)	1 (14.3%, 0.8–58.0)	0	19 (12.6%, 7.9–19.2)
Treatment parameters[†]							
OPU S-E2 (pmol/L)	4497.1 ± 7950.9	2978.0 ± 1858.6	4195.5 ± 3117.8	3259.7 ± 1991.7	2960.0 ± 1723.2	2164.8 ± 1236.3	3711.8 ± 5429.4
OPU S-progesterone (nmol/L)	36.0 ± 22.5	26.1 ± 16.7[b]	34.2 ± 16.2	35.9 ± 17.0	40.3 ± 19.2	33.5 ± 26.9	33.3 ± 19.9
Total dose of FSH (IU)	1992.0 ± 704.0	2297.1 ± 925.0	1743.8 ± 525.6	1919.6 ± 726.3	2142.9 ± 574.2	2475.0 ± 656.7	2073.9 ± 702.8
Follicular diameter (mm)	21.4 ± 2.9	21.5 ± 3.3	21.7 ± 2.0[b]	19.9 ± 2.0	20.3 ± 1.6	19.3 ± 1.1[b]	21.1 ± 2.9
Oocytes (N)	11.6 ± 7.1	9.1 ± 5.5[b]	13.6 ± 9.3	11.1 ± 6.6	9.4 ± 8.3	11.3 ± 7.4	10.9 ± 6.8
Mature oocytes (N)[d]	9.2 ± 6.0	6.9 ± 4.5[b]	9.0 ± 7.8	9.6 ± 5.3	7.7 ± 6.8	8.5 ± 6.1	8.4 ± 5.7
2 PN-stage oocytes (N)	6.6 ± 5.0	4.6 ± 3.0[b]	6.0 ± 6.2	6.3 ± 4.0	4.7 ± 3.7	3.8 ± 4.1	5.8 ± 4.3
Good-quality embryos (N)[e]	3.8 ± 3.6	2.9 ± 2.3	2.9 ± 2.9	3.6 ± 3.1	2.4 ± 2.6	1.0 ± 2.0	3.3 ± 3.1
Rate of quality embryos (%)[f]	57.3 ± 30.0	56.4 ± 32.3	46.3 ± 38.4	52.9 ± 32.7	51.7 ± 33.6	14.8 ± 25.6	54.8 ± 31.6
Transferred embryos (N)	1.8 ± 0.5	1.8 ± 0.7	1.4 ± 0.7	1.9 ± 0.5	1.6 ± 0.8	1.3 ± 1.0	1.8 ± 0.6
Status of pregnancy[‡]							
hCG negative	44 (65.7%, 53.0–76.6)	29 (65.9%, 50.0–79.1)	6 (75.0%, 35.6–95.6)	16 (69.6%, 47.0–85.9)	5 (71.4%, 30.3–94.9)	3 (75.0%, 21.9–98.7)	103 (67.3%, 59.2–74.5)
Intrauterine	17 (25.4%, 15.9–37.7)	11 (25.0%, 13.7–40.6)	2 (25.0%, 4.5–64.4)	5 (21.7%, 8.3–44.2)	2 (28.6%, 5.1–69.7)	1 (25.0%, 1.3–78.1)	38 (24.8%, 18.4–32.6)
Biochemical	5 (7.5%, 2.8–17.3)	4 (9.1%, 3.0–22.6)	0	2 (8.7%, 1.5–29.5)	0	0	11 (7.2%, 3.8–12.8)
No ultrasound performed	1 (1.5%, 0.1–9.1)	0	0	0	0	0	1 (0.7%, 0.0–4.1)
Fetuses (N[†])	0.3 ± 0.6	0.4 ± 0.7	0.3 ± 0.5	0.3 ± 0.6	0.3 ± 0.5	0.3 ± 0.5	0.3 ± 0.6

[†] Continuous variables are provided as mean ± standard deviation. [‡]Categorical variables are provided as absolute numbers (percentage, 95% confidence interval of percentage). Differences between study groups: [a]Reference group; [b]t-test, $P < 0.05$; [c]Proportion test, $P < 0.05$. Associations between different parameters assessed by adjusted regression models are provided in the text. [d]Number of oocytes which reached meiosis II stage at 4–6 h after oocyte retrieval. [e]Number of embryos with at least four blastomeres and <20% fragmentation on the second day after-ICSI. [f]The proportion (%) of good-quality embryos obtained from all 2PN fertilized oocytes. Abbreviations: hCG: human chorionic gonadotrophin; ICSI: intracytoplasmic sperm injection; OPU: oocyte pick-up day; PN: pronucleus; S-E2: serum estradiol; S-FSH: serum follicle-stimulating hormone.

Table 3: Biomarkers in the follicular fluid of patient groups.

	Male factor infertility ($N = 67$)[a]	Tubal factor infertility ($N = 44$)	Polycystic ovary syndrome ($N = 8$)	Endometriosis ($N = 23$)	Unexplained infertility ($N = 7$)	Other reasons ($N = 4$)	Total ($N = 153$)
Biomarkers (pg/mL)[†]							
G-CSF	82.5 (0–2464.0)	48.1 (0–4986.0)	104.1 (0–3156.0)	122.2 (0–4809.0)	118.5 (0–463.4)	23.7 (0–341.6)	89.7 (0–4986.0)
IL-1β	0 (0–236.8)	0 (0–53.6)	0 (0–29.0)	0 (0–110.3)	0 (0-0)	0 (0–143.1)	0 (0–236.8)
IL-6	0 (0–18.7)	0 (0–10.7)	0 (0–16.2)	0 (0–37.2)	0 (0-0)	0 (0–8.4)	0 (0–37.2)
IL-12p70	0 (0–24.9)	0 (0–6.1)	0 (0–8.1)	0 (0–21.0)	0 (0-0)[b]	0 (0–8.1)	0 (0–24.9)
IL-18	311.0 (0–722.0)	290.2 (0–812.5)	463.4 (0–648.5)	283.3 (44.6–874.3)	199.1 (0–255.5)[b]	310.9 (110.8–767.0)	297.2 (0–874.3)
IL-23	282.3 (0–1069.0)	208.7 (0–1280.0)	237.4 (0–746.4)	388.8 (0–1160.0)	408.5 (0–557.8)	120.3 (0–260.3)	260.3 (0–1280.0)
IFN-α	0 (0–150.7)	0 (0–107.6)	0 (0–93.5)	0 (0–114.2)	0 (0-0)	0 (0–161.9)	0 (0–161.9)
IFN-γ	0 (0–111.2)	0 (0–74.5)	0 (0–60.4)	0 (0–111.2)	0 (0-0)	9.5 (0–147.5)	9.5 (0–147.5)
TNF-α	0 (0–30.7)	0 (0–10.8)	0 (0–5.3)	0 (0–21.0)	0 (0-0)	0 (0–58.8)	0 (0–58.8)
IL-8	307.3 (119.4–4857.0)	367.2 (117.4–1117.0)[b]	417.6 (236.9–1032.0)	473.6 (172.8– 1879.0)[b]	424.3 (343.8–1472.0)	416.2 (172.8–2851.0)	371.2 (117.4–4857.0)
MCP-1	1019.0 (594.2–2046.0)	1054.0 (416.1–2564.0)	1067.0 (656.4–1572.0)	1033.0 (373.8–2780.0)	992.9 (818.1–1265.0)	801.7 (198.4–1598.0)[b]	1016.0 (198.4–2780.0)
MIP-1α	143.6 (0–5766.0)	80.6 (0–15990.0)	555.8 (0–19840.0)	227.6 (0–18230.0)	52.3 (0–3383.0)	136.3 (0–1788.0)	130.8 (0–19840.0)
MIP-1β	52.3 (6.0–1254.0)	48.2 (11.5–433.2)	38.7 (17.59–96.4)	51.7 (17.1–120.9)	40.7 (36.8–64.5)	63.5 (25.7–967.0)	48.4 (6.0–1254.0)
RANTES	97.4 (0–705.1)	97.4 (0–908.3)	50.1 (0–189.3)	146.6 (0–438.8)	77.4 (12.2–182.7)	74.1 (2.6–1428.0)	97.4 (0–1428.0)
sAPO-1/Fas	129.0 (0–564.2)	169.4 (0–9589.0)	152.1 (0–4469.0)	94.9 (0–520.4)	96.8 (64.3–292.5)	98.8 (0–226.8)	129.0 (0–9589.0)
CD44(var6)	8426.0 (5063.0–14030.0)	8554.0 (5348.0–20610.0)	10750.0 (6394.0–12130.0)[b]	8219.0 (5535.0–11770.0)	6619.0 (5168.0–9348.0)[b]	6836.0 (5611.0–10200.0)	8219.0 (5063.0–20610.0)

[†] Concentrations are provided as medians (minimum – maximum value). Differences between study groups: [a]Reference group; [b]Mann-Whitney U-test, $P < 0.05$. Associations between different parameters assessed by adjusted regression models are provided in the text.

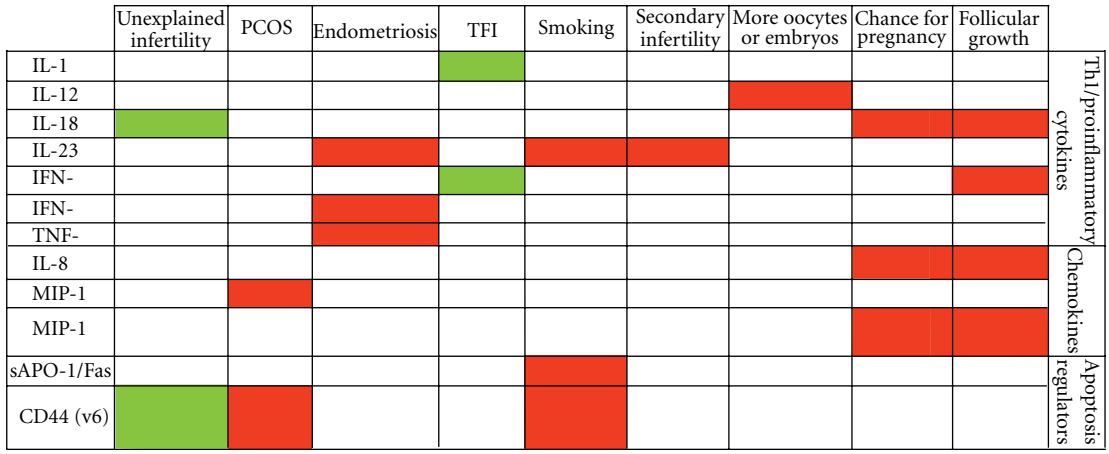

Figure 1: Associations between biomarkers and infertility parameters. Red boxes indicate positive association, green boxes negative association; empty boxes indicate no association found by adjusted regression analysis. Male factor infertility was chosen as a reference group, but in cases marked with $*$. TFI was used as a reference. Abbreviations are as mentioned in the text.

Active smoking was associated with elevated follicular CD44(v6) levels (adjusted for age and cause of infertility $r = 1227.8$ pg/mL, $P = 0.019$ versus never smokers group) and sAPO-1/Fas levels (adjusted $r = 464.9$ pg/mL, $P = 0.031$ versus never-smokers group). Similarly, follicular IL-23 levels were higher in women who reported history of smoking or current smoking, as compared to never-smokers, regardless of age or cause of infertility (adjusted $r = 107.6$ pg/mL, $P = 0.043$). In addition, an elevated IL-23 concentration was associated with women experiencing secondary infertility rather than primary infertility (regardless of the cause of infertility; adjusted $r = 94.6$ pg/mL, $P = 0.043$).

3.2. Associations between Infertility Treatment Parameters and Biomarker Levels in Follicular Fluid. A positive association was determined to exist between the concentration of follicular IL-12 and the number of fertilized oocytes (adjusted $r = 0.15$ pg/mL per every additional 2PN oocyte, $P = 0.007$) and the proportion of good-quality embryos (adjusted $r = 0.22$ pg/mL per every additional embryo, $P = 0.006$), when the data were adjusted for age, cause of infertility, and follicular size. Achieving intrauterine pregnancy was associated with higher levels of follicular MIP-1β, as compared to hCG-negative patients (adjusted for age and cause of infertility $r = 48.0$ pg/mL, $P = 0.047$). In addition, follicular MIP-1β and IFN-α levels were both positively associated with the diameter of a follicle (adjusted $r = 7.8$ pg/mL, $P = 0.037$ and $r = 2.4$ pg/mL for every millimeter in diameter, $P = 0.023$, resp.), regardless of age or cause of infertility.

The concentration of IL-8 in follicular fluid was positively associated with intrauterine pregnancy (adjusted for age, cause of infertility, rate of good-quality embryos transferred, and endometrial thickness $r = 207.5$ pg/mL, $P = 0.051$), and also with parity (adjusted for age and cause of infertility $r = 150.6$ pg/mL for every child born, $P = 0.039$). Not surprisingly, IL-8 was also associated with higher levels of serum progesterone after ovarian stimulation (adjusted $r = 4.7$ pg/mL, $P = 0.031$).

Follicular IL-18 levels appeared to be positively correlated with several outcomes, including increased chance for intrauterine pregnancy (adjusted for the cause of infertility $r = 71.6$ pg/mL, as compared to hCG-negative patients, $P = 0.054$), number of fetuses detected by ultrasonography (adjusted for age, cause of infertility, number of embryos transferred, rate of good-quality embryos among them, and endometrial thickness $r = 67.2$ pg/mL for every additional fetus, $P = 0.020$), and with increased parity (adjusted for age and cause of infertility $r = 60.7$ pg/mL for every child to give birth, $P = 0.038$). Interestingly, the levels of both follicular IL-8 and IL-18 increased as follicles grew (adjusted for age and cause of infertility $r = 40.2$ pg/mL, $P = 0.005$, and $r = 1\ 3.1$ pg/mL for every additional millimeter in diameter, $P = 0.022$, resp.).

3.3. mRNA Analysis of the Measured Protein Transcripts from MGC and CGC. Our mRNA expression analysis demonstrated that most of the studied transcripts were more abundantly expressed in MGC (Figure 2). G-CSF and sAPO-1/Fas were not differentially expressed in the two cell types. Both of the interferons examined were found to be more highly expressed in CGC, although this result was not statistically significant (Table 4). When the abundance of intracellular transcripts was analyzed, the mRNA levels were found to differ by several orders of magnitude and were characterized by substantial interpatient variability (Table 4).

4. Discussion

In the current study, we evaluated the expression of 16 different biomarkers in the follicular fluid of infertile women by using multiplex assay from Bender MedSystems. These biomarkers included Th1/proinflammatory and anti-inflammatory cytokines, chemokines and antiapoptotic biomarkers that had previously been implicated in ovarian function by our previous study [7]. Ultimately, we found

Table 4: Relative* mRNA abundance of measured proteins in cumulus and mural granulosa cells.

Biomarkers	CGC ± SD	MGC ± SD	*P* value (paired *t*-test)
G-CSF	0.000216 ± 0.000151	0.000207 ± 0.000109	0.930
IL-1β	0.001025 ± 0.000640	0.070199 ± 0.108075	0.178
IL-6	0.000082 ± 0.000059	0.003981 ± 0.006412	0.209
IL-12A	0.000104 ± 0.000047	0.000209 ± 0.000083	0.022
IL-18	0.001506 ± 0.000958	0.007472 ± 0.002346	< 0.001
IL-23A	0.000009 ± 0.000004	0.000025 ± 0.000014	0.016
IFN-α	0.000236 ± 0.000186	0.000044 ± 0.000021	0.127
IFN-γ	0.000064 ± 0.000045	0.000027 ± 0.000021	0.157
TNF-α	0.000133 ± 0.000150	0.001899 ± 0.002363	0.117
IL-8	0.022214 ± 0.009590	0.487650 ± 0.431439	0.045
MCP-1	0.003409 ± 0.004521	0.008618 ± 0.009842	0.239
MIP-1α	0.000015 ± 0.000011	0.000451 ± 0.000718	0.191
MIP-1β	0.001029 ± 0.000864	0.055073 ± 0.071566	0.122
RANTES	0.000650 ± 0.000282	0.014258 ± 0.021169	0.293
sAPO-1/FAS	0.000024 ± 0.000014	0.000024 ± 0.000016	0.979
CD44(v6)	0.000024 ± 0.000006	0.000045 ± 0.000028	0.158

* As compared to the average of three housekeeping gene transcripts: beta actin, glyceraldehyde-3-phosphate dehydrogenase, and ribosomal protein RPL13A.
Abbreviations: CGC: cumulus granulosa cells; MGC: mural granulosa cells; SD: standard deviation.

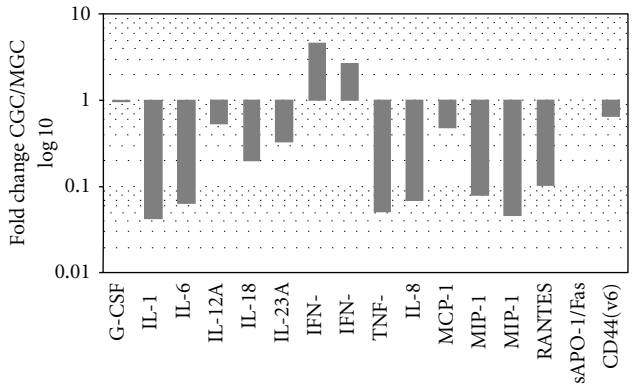

Figure 2: Differential expression of measured protein transcripts in cumulus and mural granulosa cells. ∗ Differences in expression were statistically significant. CGC: cumulus granulosa cells, MGC: mural granulosa cells.

that 12 of the 16 examined biomarkers were associated with a cause of infertility or IVF treatment outcome.

The mammalian ovulation event can be considered from the perspective of an inflammatory reaction, with proinflammatory cytokines produced and functionally interacting throughout the process [10]. For example, IL-1β has been evidenced to participate in ovulation induction by facilitating follicular rupture [27]. The fact that we found lower values of IL-1β in TFI patients indicates that impairment of folliculogenesis might have occurred and contributed to the infertility of these women. IL-18 is known to induce cytokines that are important for both folliculogenesis and ovulation, including IL-1β, TNF-α, and IFN-γ [28]. Our finding that levels of IL-18 were relatively low in unexplained infertility patients might then reflect an underlying perturbed immunological profile for this infertility cause. Importantly, IL-18 has been suggested to favor ovarian folliculogenesis; a positive correlation has been reported between the level of follicular IL-18 and the number of retrieved oocytes and implantation success in women with different etiologies of infertility [29, 30]. Our finding that follicular growth positively correlates with IL-18 levels indirectly supports the role of IL-18 in follicle maturation. Furthermore, our finding that higher follicular IL-18 was associated positively with parity indicates that this cytokine may increase the chance for pregnancy.

The elevated levels of follicular IFN-γ found in our group of endometriosis patients is in agreement with previous results obtained with serum samples [27]. Increased production of IFN-γ may reflect the immune system's efforts to overcome apoptosis inhibition and to decrease cell proliferation in the case of endometriosis [31]. Additionally, while a temporary increase in the concentration of IFN-γ seems to be essential for ovulation, IFN-γ levels that exceed normal physiologic concentrations may inhibit ovulation and contribute to early pregnancy loss [28]. IFN-α is synthesized primarily in response to infection, but the IFN-α signaling pathways have also been demonstrated to be involved in reproduction processes, even in the absence of detectable infection [12]. The fact that the group of healthy women (with male factor infertility) in our study possessed higher levels of IFN-α than did women with TFI further supports a positive role for IFN-α in reproduction. In addition, the positive correlation that was identified between follicular IFN-α levels and follicular diameter was in accordance with previous IFN-α data from preovulatory granulosa cells [32].

We observed elevated levels of TNF-α in endometriosis patients, as compared to TFI patients. It is possible that this finding simply reflects increased TNF-α serum levels

that had infiltrated into the follicular fluid [33] or increased secretion by granulosa cells induced by the inflammatory pelvic milieu in endometriosis [34]. TFI patients' expression of follicular TNF-α has also been previously suggested to be below the threshold of standard detection systems [6]. TNF-α in IVF has already been the subject of much study by infertility researchers. Some authors have concluded that follicular TNF-α might deteriorate the microenvironment in the follicle, thereby negatively affecting oocyte and embryo quality [35]. Still others have proposed a positive role of TNF-α regarding oocyte quality, and ovulation [36]. Overall, the roles of TNF-α in female reproduction are likely to be complex and dynamically involved in the different stages of folliculogenesis [37].

Previous studies examining IL-12 in the follicular fluid have yielded contradictory results. Nevertheless, a majority of the findings have indicated that IL-12 is associated with a negative effect on folliculogenesis, oocyte quality and implantation [9, 20, 38]. We failed to detect any correlation between the follicular level of IL-12 and the pregnancy outcome of IVF. Nonetheless, there was a positive association identified between IL-12 and the quality of oocytes and embryos. Our results are similar to a study published by Ostanin et al. [39], wherein the authors reported that follicular concentration of IL-12 was elevated in women who produced more high-quality oocytes. IL-12 is a Th1 cytokine that can become cytotoxic at high levels. It is, therefore, not unexpected that high concentrations of IL-12 in the follicular fluid might impair the natural process of folliculogenesis and ovulation [38]. However, in the current study, the mean concentration of IL-12 was found to be more than 10-fold lower than that reported in studies that had concluded deleterious function of IL-12 on reproduction [9, 20, 38]. Thus, we suggest a dose-dependent role for IL-12 in the follicles.

We also determined that endometriosis was associated with increased levels of follicular IL-23. Given that IL-23 is known to participate in autoimmune diseases by promoting inflammation, a hallmark of endometriosis, this result was not surprising. Impaired follicular fluid microenvironment characterized by elevated inflammatory cytokines may in fact be the cause for poor oocyte quality, which in turn could lead to poor IVF outcome in patients with endometriosis [19]. The detrimental effect of IL-23 on fecundity is further supported by our findings of higher levels of IL-23 in women who smoked or who suffered from secondary infertility.

MIP-1α is a marker for ongoing acute or chronic inflammatory host responses [18, 40]. Dahm-Kähler et al. [41] failed to detect MIP-1α in follicular fluids of unstimulated menstrual cycles, leading to their conclusion that MIP-1α is not produced under physiological conditions. Our contradictory findings of elevated levels of MIP-1α in PCOS patients may reflect a character of increased inflammation in stressed ovaries. To date, very few studies have appeared in the literature that investigate the function of MIP-1β in female reproduction, although this chemokine has been suggested to promote folliculogenesis and pregnancy establishment [39]. Such a positive role was also supported by our finding that higher follicular fluid MIP-1β levels correlated with follicular growth and achieving pregnancy.

The correlation of IL-8 concentration with follicular growth is in accordance with previously reported results. When taking into consideration that IL-8 has also been detected in unstimulated cycles [38], the involvement of this chemokine in the natural process of folliculogenesis and ovulation can be assumed [42]. Moreover, a recent study showed that lower serum levels of IL-8 correlated with a higher risk for extrauterine pregnancy [43]. Thus, our finding of higher follicular fluid IL-8 in cases of normal intrauterine pregnancy seems sensible. Nonetheless, two previous studies demonstrated no correlations between follicular fluid IL-8 concentration and IVF cycle parameters or pregnancy results [38, 42]. The discrepant results obtained from these studies and our own could be due to differences in sample sizes, patient groups examined, or detection methods used; this issue needs further investigation.

sAPO-1/Fas mediates apoptosis inhibition, which is important in preventing oocytes from succumbing to atresia during follicular maturation [21]. Increased sAPO-1/Fas levels have also been associated with enhanced activity of smoking-induced antiapoptotic signaling pathways in the oral cavity, which leads to epithelial hyperplasia [44]. In our study, we detected higher levels of sAPO-1/Fas in active smokers. Thus, our findings suggest that a compensatory increase of sAPO-1/Fas was established in the apoptosis-favoured environment of the follicles in active smokers. A similar effect has also been proposed for CD44(v6) in the ovary, where macrophage membrane-expressed CD44 protein has been shown to participates in clearance of apoptotic granulosa cells [44, 45]. Our findings of lower levels of CD44(v6) in unexplained infertility and higher levels in PCOS and active smokers might reflect impaired apoptosis mechanisms in the ovaries of these patients.

Considering that cytokines likely affect ovarian function, one could argue about the source of these immunomodulatory factors in follicular fluid. The ovulatory process is comparable to a classical local inflammatory reaction, and leukocytes have been shown to participate actively in the cyclic events of the ovary [6]. However, it is unlikely that migrating leukocytes producing proinflammatory cytokines represent the principal mechanism by which ovarian folliculogenesis is regulated [6]. Increased levels of serum-derived cytokines in follicular fluid have been demonstrated in endometriosis [33]. In addition, upregulated expression of proinflammatory cytokines by granulosa cells has been detected in cases of infertility [7]. Here, we confirmed our previous findings from the Affymetrix GeneChip platform using real-time PCR analysis to monitor mRNA expression in different conditions of infertility, as compared to levels expressed in conditions of normal fertility. To the best of our knowledge, our results represent the first description of the human granulosa cell expression profile of IL-12A, IL-23A, IL-18, MIP-1α, MIP-1ß, IFN-α, IFN-γ, and sAPO-1/FAS. MIP-1ß has been studied in the mouse cumulus-oocyte complex, where its expression increased in response to experimental exposure to hyaluronan fragments, and the related signal was determined to be mediated by Toll-like receptors [46]. On the other hand, luteinizing hormone induction of IFN-α was shown in rats and determined

to function as a modulator of steroidogenesis and MGC differentiation [47]. It is well known that cytokines and apoptosis networks functionally interact with one another in a variety of mammalian, and human, tissues. Therefore, our results also indicate a strong role of these proteins in human follicular physiology.

In conclusion, we discovered that various infertility etiologies are accompanied by distinct intrafollicular cytokine profiles. Furthermore, some of the cytokines evaluated, such as IL-12, were determined to influence oocyte fertilization and embryo quality, while others, such as IL-18, IL-8, and MIP-1β, were found to be correlated with successful pregnancy following IVF treatment. Collectively, these factors appear to be promising prognostic markers for IVF success and should be evaluated as such by future prospective studies.

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Clinical Study

Intrauterine Growth Restriction: Cytokine Profiles of Trophoblast Antigen-Stimulated Maternal Lymphocytes

Raj Raghupathy,[1] Majedah Al-Azemi,[2] and Fawaz Azizieh[3]

[1] *Department of Microbiology, Faculty of Medicine, Kuwait University, P.O. Box 24923, Kuwait 13110, Kuwait*
[2] *Department of Obstetrics & Gynecology, Faculty of Medicine, Kuwait University, P.O. Box 24923, Kuwait 13110, Kuwait*
[3] *Department of Mathematics & Biology, Gulf University for Science and Technology, Mubarak Al-Abdullah Area, West Mishref, Hawalli 32093, Kuwait*

Correspondence should be addressed to Raj Raghupathy, raj@hsc.edu.kw

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Intrauterine growth restriction (IUGR) is an important perinatal syndrome that poses several serious short- and long-term effects. We studied cytokine production by maternal peripheral blood lymphocytes stimulated by trophoblast antigens. 36 women with a diagnosis of IUGR and 22 healthy women with normal fetal growth were inducted. Peripheral blood mononuclear cells were stimulated with trophoblast antigens and levels of the proinflammatory cytokines IL-6, IL-8, IL-12, IL-23, IFNγ, and TNFα and the anti-inflammatory cytokines IL-4, IL-10, and IL-13 were measured in culture supernatants by ELISA. IL-8 was produced at higher levels by blood cells of the IUGR group than normal pregnant women, while IL-13 was produced at lower levels. IL-8, IFNγ, and TNFα were higher in IUGR with placental insufficiency than in normal pregnancy. IL-12 levels were higher and IL-10 levels were lower in IUGR with placental insufficiency than in IUGR without placental insufficiency. We suggest that a stronger pro-inflammatory bias exists in IUGR as compared to normal pregnancy and in IUGR with placental insufficiency when compared to IUGR without placental insufficiency. Several ratios of proinflammatory to anti-inflammatory cytokines also support the existence of an inflammatory bias in IUGR.

1. Introduction

Intrauterine growth restriction (IUGR) is one of the most important perinatal syndromes and is a worldwide problem. IUGR, defined as fetal growth less than the 10th percentile for gestational age [1], puts the fetus and neonate at higher risk for perinatal mortality and morbidity [2] and the child at a permanent risk for a range of disorders that include cardiovascular and renal disease, and hypertension [3]. Affected babies have a 30–50% likelihood of intrapartum hypoxic distress and a 50% risk of neonatal complications that include hypoglycemia, meconium aspiration pneumonia, and long-term growth impairment [4].

Intrauterine growth restriction is segregated into two types, IUGR *with* placental insufficiency (or asymmetric IUGR) and IUGR *without* placental insufficiency (or symmetric IUGR). IUGR without placental insufficiency is believed to be an early embryonic event, is constitutional, and is generally attributable to genetic and chromosomal abnormalities, fetal malformation, and infections. Infants of such pregnancies have both length and weight below normal for gestational age; placentas are usually small by weight, but have no other pathologies [5]. On the other hand, IUGR with placental insufficiency (asymmetric IUGR) occurs later in gestation and usually involves a more severe growth restriction of the abdomen than of the head [6]; such pregnancies usually have significant placental pathological findings. IUGR with placental insufficiency is believed to be due to maternal diseases that bring about a reduction of uteroplacental blood flow [6].

Despite the delineation of several of the causes and risk factors of IUGR (5–20% due to chromosomal abnormalities, 5–20% due to maternal and fetal vascular disorders and infections [6]), a definite cause of IUGR is not identified

in 40–50% of all cases [7]. Logically an insufficient blood flow to the placenta is the first abnormality to suspect and indeed a significant proportion of IUGR cases is associated with placental findings, pointing to problems in fetoplacental circulation [8]. Indeed, the lack of sufficient transport of nutrients and oxygen to the fetus is commonly recognized as leading to IUGR [8], but in a number of cases restricted growth cannot be explained by placental insufficiency alone [8]. In addition to the genetic and constitutional disorders mentioned above, it is appropriate to look at possible immunologic events that may lead to IUGR with and without placental sufficiency.

Maternal immunologic factors such as cytokines, natural killer (NK) cells, activated macrophages, and lymphocytes have been shown to be associated with several pregnancy complications such as recurrent spontaneous miscarriage, preeclampsia, and preterm delivery. Cytokines have been shown to play vital roles in normal pregnancy both in the maintenance of placental growth and in the modulation of maternal immune reactivity to prevent rejection of the conceptus [9, 10]. The maternal immunologic state that is most conducive to successful pregnancy is maintained by local secretion of T helper-2 (Th2) cytokines and some types of pregnancy complications seem to be associated with a predominance of T helper-1 (Th1) reactivity in the mother; this appears to be the case for recurrent spontaneous miscarriage [11–13], preterm delivery [14, 15], and preeclampsia [16, 17].

Th1 and Th2 cells are two of the major subsets of $CD4^+$ T-helper cells; they have different cytokine production profiles and accordingly different roles in immune responses. Th1 cells secrete the proinflammatory cytokines IL-2, IFNγ, TNFα, and TNFβ which activate macrophages and cell-mediated reactions relevant to cytotoxic reactions and delayed-type hypersensitivity [18, 19]. Th2 cells secrete IL-4, IL-5, IL-10, and IL-13 which induce vigorous humoral immunity [18, 19]. Th1 cytokines tend to be inflammatory cytokines, while some of the Th2 cytokines tend to have anti-inflammatory properties.

While there are numerous studies on cytokine profiles in pregnancy complications like recurrent miscarriage, preterm delivery, and pre-eclampsia [9–17, 20–22], immunological studies in IUGR are relatively small in number. There are few reports on cytokine levels in IUGR. Some studies have estimated cytokines in serum and amniotic fluid, but none have yet focused on cytokine production by maternal lymphocytes. We stimulated maternal peripheral blood mononuclear cells from IUGR pregnancies and normal pregnancies with a trophoblast antigen extract and examined the resulting cytokine production pattern to explore possible relationships between cytokines and IUGR with placental insufficiency and without placental insufficiency.

2. Materials and Methods

2.1. Subjects. This study has the approval of the Ethics Committee of the Faculty of Medicine, Kuwait University; healthy pregnant women (controls) and subjects with IUGR were inducted into this study after informed consent was obtained from them. Subjects were enrolled at two high-risk pregnancy clinics at Kuwait Maternity Hospital, a tertiary center. Consecutive cases with IUGR were enrolled into the study. All subjects gave informed consent. This prospective study included 36 women with a diagnosis of IUGR and 22 control healthy women with normal fetal growth attending the antenatal clinic at Kuwait Maternity Hospital (Table 1). Power analysis, conducted using the G*Power statistics program (http://www.psycho.uni-duesseldorf.de/abteilungen/aap/gpower3) [23] based on median levels of cytokines measured in our previous studies on cytokines in pregnancy [13, 17, 21], indicated that these sample numbers are adequate to demonstrate differences at the 95% confidence interval.

Early ultrasound scan was conducted on all subjects to confirm gestational age; inclusion criteria for the IUGR group were fetuses with less than 10th centile abdominal circumference. The 36 women in the IUGR group were further subdivided into 19 IUGR pregnancies with placental insufficiency and 17 IUGR pregnancies without placental insufficiency by assessment of fetal anatomy and biometry, amniotic fluid dynamics, uterine, umbilical, and fetal middle cerebral artery Doppler. Blood velocity waveforms from both uterine arteries, the umbilical artery and the fetal middle cerebral artery, were measured using duplex pulsed-wave Doppler Ultrasound Scanner (ALOKA SSD-650) with 3.5-MHZ convex transducer. Pulsatility Index was calculated as (Systolic/Diastolic)/Systolic as described in [24]. Placental insufficiency was diagnosed if pulsatility index in the umbilical artery was raised, with either absent or reversed end diastolic flow. Doppler measurements were performed by a single investigator.

The control group consisted of 22 women who had a history of at least two previous successful pregnancies with no previous spontaneous miscarriage, pre-eclampsia, preterm labor or IUGR.

2.2. Isolation of Peripheral Blood Mononuclear Cells. Five mL of venous blood samples were taken from all subjects within 24 hours of delivery. Peripheral blood mononuclear cells (PBMC) were separated from the blood samples by Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, suspended in RPMI medium (GIBCO, Auckland, New Zealand) containing 10% fetal calf serum, aliquoted into 96-well tissue culture plates at a density of 10^5 cells per well and then challenged with the trophoblast antigen extract as described below.

2.3. Trophoblast Antigen Stimulation of PBMC. Trophoblast antigen extracts were prepared as described previously [20–22] from the human gestational choriocarcinoma cell line JEG-3 (American Type Culture Collection, Md, USA), which is of trophoblastic origin. JEG cells were cultured in RPMI-1640 medium until 80% confluence is reached, harvested without trypsinization using a rubber cell scraper, washed three times in medium and then disrupted in a Dounce homogenizer (~100 strokes). The suspension was then centrifuged at 3000 rpm for 10 minutes, the supernatant filtered

Table 1: Demographic data on subjects in this study.

	Control $N = 22$	IUGR $N = 36$	P value	IUGR with placental Insufficiency $N = 19$	IUGR without placental Insufficiency $N = 17$	P value
Maternal age	32.4 ± 4.2	35.1 ± 3.7	NS	34.6 ± 3.3	36.1 ± 4.3	NS
Mode of delivery						
C.S.	6	15	—	9	6	—
S.V.D	16	21		10	11	
Outcome						
Preterm	2	8	—	6	2	—
Term	20	28		13	15	
Birthweight (Kg)	3.6 ± 1.2	2.3 ± 0.7	<0.001	2.0 ± 0.9	1.9 ± 0.6	NS

NS: Nonsignificant; C.S.: Caesarian section; S.V.D.: Single vaginal delivery.

through a 0.20 μM filter, aliquoted and stored at −20°C until use. This material was used to stimulate maternal peripheral blood cells. Maternal PBMCs were stimulated at a density of 10^5 cells per well with JEG antigen. Initial standardization experiments in our laboratory (data not shown) showed that the optimal concentration for cell proliferation upon stimulation was 30 μg/mL. PBMCs were cultured for 4 days after antigen stimulation, after which supernatants were collected for cytokine estimation.

2.4. Determination of Cytokine Levels by ELISA. Levels of the proinflammatory cytokines IL-6, IL-8, IL-12, IL-23, IFNγ, and TNFα and the anti-inflammatory cytokines IL-4, IL-10 and IL-13 in trophoblast antigen-stimulated cell culture supernatants were measured by ELISA. Kits for estimating IL-4, IL-8, IL-10, IL-12, IFNγ and TNFα were obtained from Beckman-Coulter (Marseilles, France), IL-13 kits from R & D Systems (Minneapolis, Minn, USA) and IL-23 kits from Bender Medsystems (Vienna, Austria). Sensitivities of the kits and the reproducibilities within and between assays are provided in the appendix below. The manufacturer's protocols were followed for these assays which are based on the antibody sandwich principle. Samples were tested in triplicate and absorbance values read using an ELISA Reader. Accurate sample concentrations of cytokines were determined by comparing their respective absorbancies with those obtained for the reference standards plotted on a standard curve.

2.5. Statistical Analyses. The standard Mann-Whitney-U test was used for nonparametric comparisons of median cytokine levels, as the data were not normally distributed. Differences were considered significant if the P value ≤0.05.

3. Results

We stimulated maternal PBMC with the trophoblast antigen extract and then measured the levels of the proinflammatory cytokines IL-6, IL-8, IL-12, IL-23, IFNγ and TNFα and the anti-inflammatory cytokines IL-4, IL-10 and IL-13. Median levels of cytokines were compared for statistical significance.

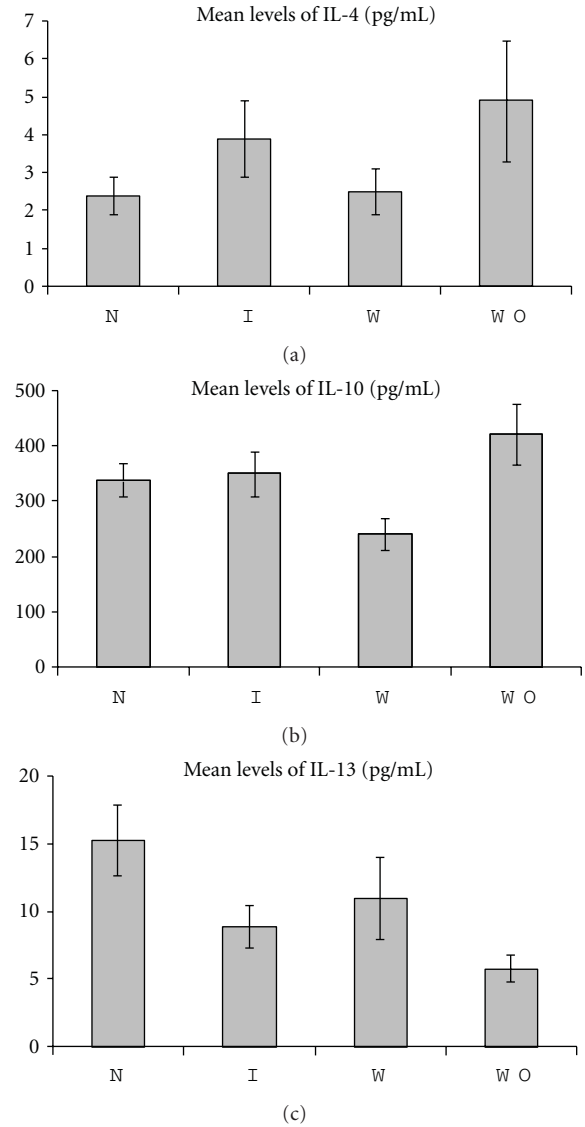

Figure 1: Mean levels of the anti-inflammatory cytokines IL-4, IL-10, and IL-13 produced by PBMC from normal pregnancy (*N*), all IUGR subjects (*I*), IUGR with placental insufficiency (*W*), and IUGR without placental insufficiency (*WO*).

We also calculated the means of ratios of proinflammatory to anti-inflammatory cytokines (e.g., IFNγ/IL-4, IL-8/IL-10). This was done to determine whether bias or dominance of pro- or anti-inflammatory cytokines exists in the stimulated cultures. The following groups were compared statistically: IUGR versus normal pregnancy, IUGR with placental insufficiency versus normal pregnancy, IUGR without placental insufficiency versus normal pregnancy and finally IUGR with placental insufficiency versus IUGR without placental insufficiency.

3.1. Comparison of Cytokine Profiles in IUGR versus Normal Pregnancy. We found significantly higher levels of the proinflammatory cytokine IL-8 (mean ± SEM = 1780 pg/mL ± 44) in IUGR (i.e., all IUGR pregnancies) as compared to normal pregnancy (mean ± SEM = 1049 pg/mL ± 45) ($P < 0.0001$) (Figure 2). We also found significantly lower levels of the anti-inflammatory cytokine IL-13 in IUGR (8.9 pg/mL ± 1.6) versus normal pregnancy (15.3 pg/mL ± 2.6) ($P < 0.02$) (Figure 1). The IL-8/IL-13 ratio is also higher in IUGR as compared to normal pregnancy ($P < 0.0005$). Other cytokine ratios which are significantly higher in IUGR than in normal pregnancy are IL-12/IL-13 ($P < 0.02$), IL-6/IL-13 ($P < 0.01$) and TNFα/IL-13 ($P < 0.02$) (Table 2). Other cytokine ratios were not significantly different between IUGR and normal pregnancy. Based on the higher levels of IL-8, the lower levels of IL-13 and the higher mean cytokine ratios mentioned above, we suggest that a proinflammatory cytokine pattern exists among PBMC from IUGR subjects. However, we found higher levels of the proinflammatory cytokine IL-23 in normal pregnancy (479 pg/mL ± 15) than in IUGR (356 pg/mL ± 13) ($P < 0.0001$). The IL-23/IL-4 ($P < 0.003$) and IL-23/IL-10 ($P < 0.005$) ratios are also higher in IUGR versus normal pregnancy.

3.2. Comparison of Cytokine Profiles in IUGR with Placental Insufficiency versus Normal Pregnancy. The levels of the proinflammatory cytokines IL-8 (1803 pg/mL ± 89, $P < 0.001$), IFNγ (126 pg/mL ± 33, $P < 0.02$), and TNFα (340 pg/mL ± 46, $P < 0.04$) are significantly higher in IUGR with placental insufficiency as compared to normal pregnancy (1049 pg/mL ± 45, 18 pg/mL ± 6, 70 pg/mL ± 21, resp.). The IL-12/IL-13 and IL-12/IL-10 ratios are significantly higher in IUGR with placental insufficiency when compared to normal pregnancy ($P < 0.04$ in both cases) (Table 2). The higher ratios and the higher levels of IL-8, IFNγ, and TNFα are suggestive of a higher proinflammatory bias in IUGR with placental insufficiency than in normal pregnancy. None of the other cytokines, except for IL-23 (Figure 3), and none of the other ratios, except for IL-23/IL-4 were significantly different; IL-23 levels were significantly higher in normal pregnancy (479 pg/mL ± 15) versus IUGR with placental insufficiency (350 pg/mL ± 24) ($P < 0.0001$) and the IL-23/IL-4 ratio was also higher in normal pregnancy ($P < 0.01$).

3.3. Comparison of Cytokine Profiles in IUGR without Placental Insufficiency versus Normal Pregnancy. The proinflammatory cytokine IL-8 is produced at higher levels by PBMC from IUGR without placental insufficiency (1793 pg/mL ± 33) than by PBMC from normal pregnant controls (1049 pg/mL ± 45) ($P < 0.0001$). On the other hand, the anti-inflammatory cytokine IL-13 is produced at lower levels by PBMC from IUGR without placental insufficiency (5.8 pg/mL ± 1) than by PBMC from normal pregnant controls (15.3 pg/mL ± 2.6) ($P < 0.002$) (Figures 1 and 2). Two of the ratios, IL-6/IL-13 ($P < 0.006$) and IL-8/IL-13 ($P < 0.001$), were significantly higher in IUGR without placental insufficiency compared to normal pregnancy. However, the IFNγ/IL-10 ratio was actually higher in normal pregnancy than in IUGR without placental insufficiency ($P < 0.03$) (Table 2). The higher IL-8 levels and the lower IL-13

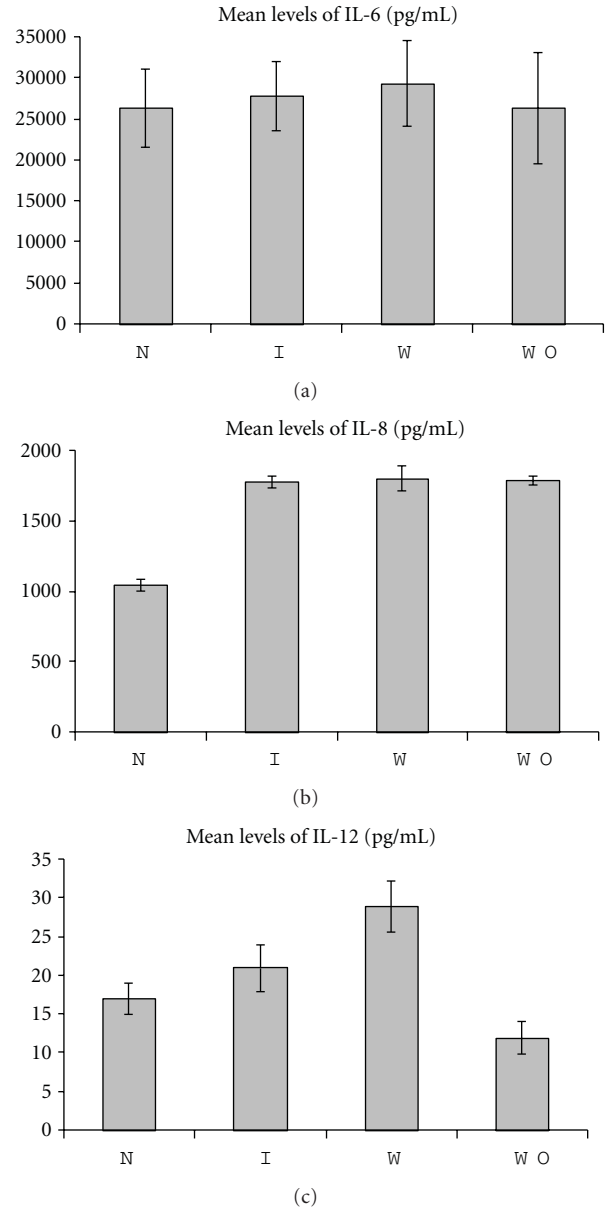

Figure 2: Mean levels of the proinflammatory cytokines IL-6, IL-8, and IL-12 produced by PBMC from normal pregnancy (*N*), all IUGR subjects (*I*), IUGR with placental insufficiency (*W*), and IUGR without placental insufficiency (*WO*).

Table 2: Means of ratios of proinflammatory to anti-inflammatory cytokines. All possible combinations of pro- and anti-inflammatory cytokines were compared, but only the ones which are significantly different are presented in this table. $I > N$ indicates that the ratio is higher in IUGR than in normal pregnancy, $WO > N$ indicates that the ratio in IUGR without placental insufficiency subgroup is higher than in normal pregnancy group, and so on.

Cytokine ratio	Normal pregnancy control (N)	Total IUGR (I)	IUGR with placental insufficiency (W)	IUGR without placental insufficiency (WO)	Significant differences
IL-6/IL-13	3265	18134	7142	22189	$I > N$ $WO > N$
IL-8/IL-10	6	7	9	4	$W > WO$
IL-8/IL-13	197	1169	526	1285	$I > N$ $WO > N$
IL-12/IL-4	14	17	26	10	$W > WO$
IL-12/IL-10	0.04	0.08	0.13	0.04	$W > N$ $W > WO$
IL-12/IL-13	1.4	6	4	8	$I > N$ $W > N$
IFNγ/IL-10	0.14	0.23	0.44	0.09	$N > WO$
TNFα/IL-13	11	210	91	101	$I > N$
IL-23/IL-4	357	248	210	273	$N > I$ $C > W$ $N > WO$
IL-23/IL-10	23	1.4	1.7	1.2	$N > I$ $N > WO$
IL-23/IL-13	58	230	99	238	$N > WO$

levels suggest that there appears to be a shift towards a proinflammatory bias. As in the two comparisons mentioned above, IL-23 levels were significantly higher in normal pregnancy (479 pg/mL ± 15) than in IUGR without placental insufficiency (361 pg/mL ± 13) ($P < 0.0001$) as were the ratios of IL-23/IL-4, IL-23/IL-10, and IL-23/IL-13 ($P < 0.03$, $P < 0.01$, and $P < 0.03$, resp.).

3.4. Comparison of Cytokine Profiles in IUGR with and without Placental Insufficiency. The proinflammatory Th1-inducing cytokine IL-12 is produced at higher levels in IUGR with placental insufficiency (29 pg/mL ± 3.3) than in IUGR without placental insufficiency (12 pg/mL ± 2.1) ($P < 0.01$). On the contrary, the anti-inflammatory Th2 cytokine IL-10 is produced at lower levels in IUGR with placental insufficiency (240 pg/mL ± 29) as compared to IUGR without placental insufficiency (421 pg/mL ± 55) ($P < 0.01$). None of the other cytokines are significantly different. Three of the proinflammatory : anti-inflammatory cytokine ratios are higher in IUGR with placental insufficiency; these are IL-12/IL-10 ($P < 0.005$), IL-12/IL-4 ($P < 0.02$), and IL-8/IL-10 ($P < 0.01$). We infer from this data that a stronger proinflammatory cytokine bias exists in IUGR with placental insufficiency as compared to IUGR without placental insufficiency.

4. Discussion

This study was undertaken with the expectation that studies of this sort may lead to the identification of immunologic etiologies of fetal growth restriction or to immune-mediated pathophysiologic mechanisms that could lead to fetal growth restriction even if the initial etiology is nonimmunologic. While previous studies have reported the estimation of cytokine levels in the serum of women with IUGR, this is the first to present data on cytokine production profiles of maternal lymphocytes after stimulation with trophoblast antigens. T lymphocytes can be activated with mitogen, anti-CD3, and with antigens; in this study we chose to stimulate maternal T lymphocytes in PBMC with trophoblast antigens. The trophoblast cell line, JEG-3, used to prepare a trophoblast antigen extract has characteristics similar to early normal human trophoblast cells, including invasive characteristics, endocrine, and antigenic features. Previous studies using antigen extracts from this cell line [20–22] demonstrated higher Th1-type reactivity and lower Th2-type to trophoblast antigens in women with unexplained recurrent miscarriage as compared to women with a history of normal pregnancy.

We found interesting differences in the levels of some pro- and anti-inflammatory cytokines between IUGR and normal pregnancy and between IUGR with and without placental insufficiency.

The proinflammatory chemotactic cytokine IL-8 is consistently produced at significantly higher levels in IUGR subjects as a group when compared to normal pregnancy, and also in IUGR with placental insufficiency and IUGR without placental insufficiency as compared to normal pregnancy. IL-8 is induced by a variety of stimuli that include lipopolysaccharide, live bacteria, and other proinflammatory

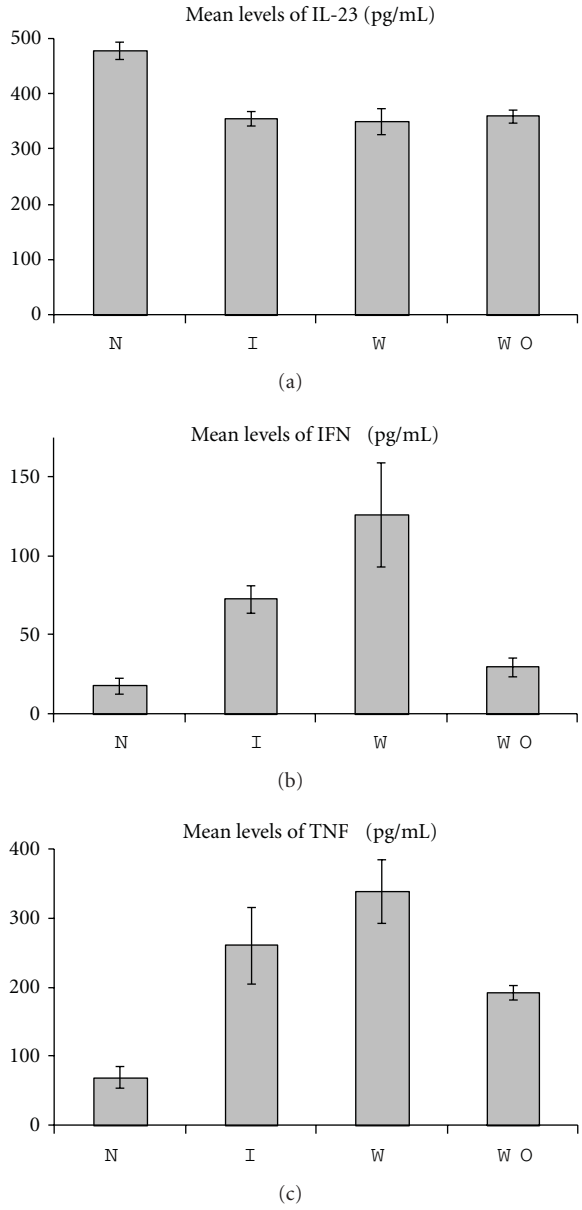

Figure 3: Mean levels of the proinflammatory cytokines IL-23, IFNγ, and TNFα produced by PBMC from normal pregnancy (*N*), all IUGR subjects (*I*), IUGR with placental insufficiency (*W*), and IUGR without placental insufficiency (*WO*).

cytokines such as TNF and IL-1 [25] and it, in turn, induces chemotaxis of inflammatory cells. It is the principal recruiter of neutrophils, the signature cell of acute inflammatory responses. In addition to recruiting cells to the site of inflammation, IL-8 also retains cells once they have arrived and stimulates neutrophils to a higher state of activation [25]. IL-8 is relatively unique in that it is produced early in the inflammatory response but persists for a prolonged period of time, unlike other proinflammatory cytokines that are usually made and cleared in a matter of hours in vivo. IL-8 is thus a key inducer and sustainer of local tissue inflammation [26].

Increased maternal and umbilical cord serum levels of IL-8 were recently shown to be higher in pre-eclampsia complicated by IUGR than in pre-eclampsia with normal fetal growth [27, 28]. However, this was not reflected in a study by Johnson et al. [29] who found no differences in the levels of IL-8 in IUGR versus normal pregnancy. Hahn-Zoric et al. [30] found higher placental levels of IL-8 in IUGR compared with appropriately developed neonates. It has been suggested that local action of cytokines like IL-8 may be responsible for the increased infiltration of macrophages that are seen in IUGR, and activated macrophages could contribute to placental dysfunction [31].

In addition to the higher production of IL-8 by PBMC from women with IUGR, the proinflammatory cytokines IFNγ and TNFα are also produced at higher levels in IUGR with placental insufficiency versus normal pregnancy. IFNγ and TNFα are the prime culprits in the development of chronic inflammation [32] and both of them are cytotoxic cytokines that induce apoptosis of target cells. IFNγ, a classical Th1 cytokine, is a crucial inducer of Th1 development and affects the activation and function of a variety of cells that include T cells, B cells, macrophages, and NK cells. TNFα is one of the most prominent inflammatory mediators and initiates inflammatory reactions of the innate immune system, including the induction of cytokine production, activation, and expression of adhesion molecules and thrombosis [33]. Along with IL-1 and IL-6, TNFα induces many of the localized changes seen in acute inflammatory reactions such as increased vascular permeability, induction of chemokine production, and the expression of adhesion molecules on vascular endothelia.

Neta et al. [34] reported that lower levels of IFNγ were associated with a reduced risk of small-for-gestational age babies and suggest that lower levels of IFNγ could indicate impairment of trophoblast function leading the authors to support a protective role for IFNγ. This is in contrast to our observation that IFNγ is produced at higher concentrations by PBMC from women with IUGR with placental insufficiency.

While there appear to be differences in observations on IFNγ in IUGR, evidence for an association between TNFα and IUGR seems to be compelling. Increased levels of TNFα have been reported in the serum of pregnancies complicated with IUGR [35]. Amarilyo et al. [36] showed higher levels of TNFα in the cord blood of IUGR infants and suggest that a state of inflammation exists in such infants. TNFα levels in maternal and umbilical cord serum are reported to be higher in pre-eclampsia complicated by IUGR than in pre-eclampsia with normal fetal growth [25]. Holcberg et al. [37] found that increased TNF secretion in placentas of IUGR fetuses is related to enhanced vasoconstriction of the fetal placental vascular bed, and Rogerson et al. [38] reported that placental TNFα levels are increased in low birth weight infants associated with malaria.

Overproduction of TNFα and other proinflammatory cytokines has been proposed to be important in the development of fetal growth restriction in response to hypoxia [39], possibly by decreasing amino acid uptake by the fetus [40].

TNFα has other effects on the placenta that may be relevant; it inhibits the growth of the trophoblast [41], interferes with placental development and invasion of the spiral arteries, is directly toxic to endothelium, and may damage the decidual vasculature [42]. TNF interferes with the anticoagulant system and may induce placental thrombosis [43]. Holcberg et al. [37] found that increased TNF secretion in placentas of IUGR fetuses is related to enhanced vasoconstriction of the fetal placental vascular bed.

Perhaps the most likely mechanism by which TNFα may contribute to IUGR is by causing apoptosis of trophoblast cells. Trophoblast cells of pregnancies with IUGR are more sensitive to apoptosis in response to cytokines and hypoxia when compared to trophoblast cells from normal pregnancies, and it is speculated that this dysregulated apoptosis may lead to the placental dysfunction seen in IUGR [44]. The apoptotic effect of TNFα is well known; it has been shown to kill trophoblast cells [45], and it is likely that the increased apoptosis in IUGR is due in part to cytokines like TNFα [46]. In fact, IUGR has been shown to be characterized by enhanced trophoblast apoptosis, and this has been suggested to lead to abnormal placentation, inadequate spiral artery remodelling, and uteroplacental vascular insufficiency [47].

If proinflammatory cytokines, such as IL-8 and TNFα, pose the risk of adverse outcomes of pregnancy, presumably these may have to be countered by anti-inflammatory cytokines. Indeed, the levels of the anti-inflammatory cytokine IL-13 are higher in normal pregnancy as compared to the IUGR group and to IUGR without placental insufficiency; we also observed a trend towards lower IL-13 levels in IUGR with placental insufficiency ($P < 0.059$). IL-13 is a Th2 cytokine with anti-inflammatory properties. IL-13 inhibits the production of the inflammatory cytokines IL-6, IL-12, TNFα, and IL-8, prevents pathological inflammation at mucosal surfaces, and inhibits cytotoxicity [48]. The enhanced levels of IL-13 in normal pregnancy versus IUGR may reflect a stronger Th2 bias or an anti-inflammatory cytokine bias in normal pregnancy. Further, Dealtry et al. [49] demonstrated the expression of IL-13 by human trophoblast cells and suggest that IL-13 may play important roles in maternal-fetal dialogue that aids in the establishment and maintenance of the placenta. Thus, the decreased levels of IL-13 production in IUGR observed in this study may be pertinent.

In addition to the proinflammatory bias in IUGR suggested by elevated levels of IL-8 and decreased levels of IL-13, a comparison of pro- to anti-inflammatory cytokines is also interesting. Ratios of IL-6/IL-13, IL-8/IL-13, IL-12/IL-13, and TNFα/IL-13 are all significantly higher in the IUGR group compared to normal pregnancy (Table 2). The IL-12/IL-13 and IL-12/IL-10 ratios are higher in IUGR with placental insufficiency, also suggestive of a stronger inflammatory skew in IUGR with placental insufficiency.

Pregnancy has been suggested to bring about a mild state of inflammation [50], and Li and Huang [31] speculate that exaggerated or excessive inflammation could result in adverse outcomes such as IUGR via a vicious cycle of coagulation, thrombosis, and inflammation. Thus, mutually enhancing cascades of coagulation and inflammation may be part of the etiopathogenesis of IUGR.

One of the objectives of this study was to compare IUGR with and without placental insufficiency. IUGR without placental insufficiency is, generally, due to constitutional causes in the absence of obvious placental pathologies, while IUGR with placental insufficiency manifests with significant placental pathology and decreased maternal-fetal blood flow. This led us to speculate that IUGR pregnancies with placental insufficiency may have a predominant proinflammatory cytokine bias. Our data suggests that this might indeed be the case. IL-12 levels are significantly higher in IUGR with placental insufficiency (Figure 2), while IL-10 levels are significantly lower (Figure 1). Three of the ratios are also higher in IUGR without placental insufficiency: IL-12/IL-10, IL-12/IL-4, and IL-8/IL-10. None of the other cytokine ratios were significantly different between the two subgroups. While this study should have ideally included cases of non-IUGR with placental insufficiency, our data suggests that there is a stronger tilt towards proinflammatory cytokines in IUGR *with* placental insufficiency than in IUGR *without* placental insufficiency.

The lower-level of IL-10 in IUGR with placental insufficiency is interesting as it is perhaps the most important anti-inflammatory cytokine found within the human immune response. It inhibits Th1 cytokine release, NF-κB signaling, expression of HLA class II molecules, macrophage, and dendritic cell function [51]. As IL-10 has profound anti-inflammatory properties, the decreased levels of IL-10 in IUGR with placental insufficiency, may be indicative of a lower proinflammatory bias in this subgroup versus IUGR without placental insufficiency subgroup. Previous studies have shown decreased levels of IL-10 in the placentas of IUGR pregnancies and this has been suggested to be relevant to the pathogenesis of IUGR [30]. Given its ability to inhibit the synthesis of proinflammatory cytokines and macrophage activity and its role in reducing apoptosis [52], IL-10 may, in part, be responsible for the maintenance of a balance against a proinflammatory bias in normal pregnancy.

IL-23 levels in this study present an interesting conundrum; we found significantly higher-levels of IL-23 in normal pregnancy as compared to the three IUGR groups in trophoblast antigen-stimulated cultures. IL-23 is known to have many similarities to IL-12. Along with IL-12, IL-23 plays an important role in bridging innate and acquired immune responses and causes multiorgan inflammation with elevated expression of inflammatory cytokines like TNFα and IL-1 [53]. It is not immediately apparent how lower levels of IL-23 are related to the pathogenesis of IUGR, but there are a few interesting leads. IL-23 is not required for Th1 responses and it appears to act not via the Th1 pathway but along the IL-23/IL-17 pathway of inflammatory responses; in fact the addition of IL-23 to murine T-cell cultures pushes Th development away from Th1/IFNγ differentiation [53]. Remarkably enough, IL-23 has been proposed to actually offer protection against the deleterious effects of TNF in implantation, explaining embryo survival in a TNF-rich environment [54]. Also, Vujisić et al. [55] reported significantly higher levels of IL-23 in the follicular fluid taken

from follicles containing oocytes, when compared with those without an oocyte; these authors propose that increased concentrations of IL-23 in follicles containing oocytes may indicate a beneficial role for this cytokine in reproduction. Our observation of lower levels of IL-23 in IUGR samples seems to support the idea of a beneficial role for IL-23 in normal pregnancy.

Based on the Th1 shift reported in recurrent miscarriage, preterm labor, and pre-eclampsia, our initial premise was to ascertain whether a similar Th1 bias exists in IUGR. In a murine model of fetal growth restriction, induced by Porphyromonas gingivalis infection, Lin et al. [56] showed this bacterium adversely affects normal fetal development via direct placental invasion and induction of fetus-specific placental immune responses characterized by a proinflammatory Th1-type cytokine profile. They found that mRNA levels of IFNγ and IL-2 were significantly increased in placentas of fetuses with growth restriction, while expression of IL-10 was significantly decreased in the same group. The authors concluded that fetal growth restriction in this model is associated with a shift in the placental Th1/Th2 cytokine balance. We do not observe an obvious Th1/Th2 bias in the cytokine production profiles of maternal PBMC; so we suggest that it is more likely that a general proinflammatory, rather than a more specific Th1-bias, operates in IUGR. This contention is based on the lack of a predominance of Th1/Th2 cytokines such as IFNγ and IL-4. However, our comparison of cytokine profiles in IUGR with and without placental insufficiency showed elevated production of IL-12 and decreased production in IUGR with placental insufficiency; IL-12 is a Th1-inducing cytokine, while IL-10 is a Th2-type cytokine and it is tempting to suggest the possibility of a Th1-bias in IUGR with placental insufficiency when compared to IUGR without placental insufficiency.

5. Conclusions

IUGR is a serious obstetric problem and it is important that its etiologies and pathogenetic mechanisms be elucidated. Identifying possible associations between IUGR and immunological effectors such as cytokines will help us understand the pathophysiology of this disease and define markers that can predict this condition. Understanding immunological mechanisms of normal pregnancy and of complications such as IUGR could lead to the development of regimens to improve fetal growth and development.

This study suggests that a proinflammatory cytokine bias exists in maternal peripheral blood mononuclear cells of women with IUGR when compared to normal pregnancy. It also supports the notion of a stronger proinflammatory tilt in IUGR with placental insufficiency as compared to IUGR without placental insufficiency. This conclusion is based on levels of cytokines produced by maternal peripheral blood cells as well as calculated ratios of pro- to anti-inflammatory cytokines. Future research should enable the elucidation of the roles of cytokines in the pathophysiology of IUGR as well as the development of new therapies that will aid the management of this condition.

Appendix

The nine ELISA kits were specific for the target human cytokine with no cross-reactivity or interference with other cytokines or cytokine receptors.

The IL-4 kit has a sensitivity of 5 pg/mL. Coefficient of variation (CV) for intra-assay precision ranged between 2.1 and 5.6%, while interassay CVs ranged from 4.8% to 9.7%.

The IL-6 kit has a sensitivity of 3 pg/mL, intra-assay CVs ranged between 1.6% and 6.8%, while interassay CVs ranged from 7.9% to 11.6%.

The sensitivity of the IL-8 kit is 8 pg/mL; the intra-assay CVs were between 2.3% and 5.5% and interassay CVs ranged from 7.6% to 10.1%.

IL-10 was measured at a sensitivity of 5 pg/mL, intra-assay CVs ranged between 3.3% and 4%, while interassay CVs were between 5.6% and 8.6%.

The IL-12 ELISA kit measures the IL-12 heterodimer, has an intra-assay CV of 5.5% and interassay CV of 10%.

The sensitivity of the IL-13 ELISA kit is 0.7 pg/mL, intra- and interassay CVs are 6% and 4.6%, respectively.

The human IL-23 Platinum ELISA measures the p19 subunit and has a sensitivity of 4 pg/mL, intra-assay CV is 5.9%, interassay CV is 6.3%.

IFNγ was measured at a sensitivity of 0.08 IU/mL, intra-assay CVs ranged between 2.2% and 12.6% and interassay CVs ranged from 6.2% to 12.2%.

The sensitivity of the TNFα kit is 5 pg/mL; intra-assay CVs for this kit were between 1.6% and 10% while interassay CVs were between 5.4% and 12.8%.

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Research Article

The Development of Severe Neonatal Alloimmune Thrombocytopenia due to Anti-HPA-1a Antibodies Is Correlated to Maternal *ABO* Genotypes

Maria Therese Ahlen,[1,2] Anne Husebekk,[1,2] Mette Kjær Killie,[1] Jens Kjeldsen-Kragh,[3,4] Martin L. Olsson,[5] and Bjørn Skogen[1,2]

[1] *Department of Laboratory Medicine, University Hospital of North Norway, 9038 Tromsø, Norway*
[2] *Department of Immunology, Institute of Medical Biology, University of Tromsø, 9037 Tromsø, Norway*
[3] *Department of Immunology and Transfusion Medicine, Oslo University Hospital, Ullevål, 0407 Oslo, Norway*
[4] *Faculty Division Ullevål University Hospital, University of Oslo, 0407 Oslo, Norway*
[5] *Division of Hematology and Transfusion Medicine, Department of Laboratory Medicine, Lund University, SE-221 00 Lund, Sweden*

Correspondence should be addressed to Maria Therese Ahlen, therese.ahlen@unn.no

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Background. Maternal alloantibodies against HPA-1a can cross placenta, opsonize foetal platelets, and induce neonatal alloimmune thrombocytopenia (NAIT). In a study of 100, 448 pregnant women in Norway during 1995–2004, 10.6% of HPA-1a negative women had detectable anti-HPA-1a antibodies. *Design and Methods*. A possible correlation between the maternal ABO blood group phenotype, or underlying genotype, and severe thrombocytopenia in the newborn was investigated. *Results*. We observed that immunized women with blood group O had a lower risk of having a child with severe NAIT than women with group A; 20% with blood group O gave birth to children with severe NAIT, compared to 47% among the blood group A mothers (relative risk 0.43; 95% CI 0.25–0.75). *Conclusion*. The risk of severe neonatal alloimmune thrombocytopenia due to anti-HPA-1a antibodies is correlated to maternal *ABO* types, and this study indicates that the observation is due to genetic properties on the maternal side.

1. Introduction

Foetal-maternal incompatibility in the human platelet antigen (HPA)-1 alloantigen system is the most common underlying cause of neonatal alloimmune thrombocytopenia (NAIT), a condition where maternal alloantibodies opsonize foetal platelets during pregnancy and reduce their survival in circulation. The incompatibility is based on a single-nucleotide polymorphism (SNP) which results in a leucine/proline substitution at residue 33 in the β3 integrin that constitutes membrane glycoprotein β3 [GPIIIa] present on platelets in complex with αIIb integrin [GPIIb] [1]. On platelets, the αIIbβ3 [GPIIb/IIIa] is also the major carrier of blood group A antigen [2].

About 10% of HPA-1a negative women who have been pregnant with an HPA-1-incompatible child have detectable HPA-1a antibodies [3]. In several studies, a correlation between maternal antibody level and the severity of thrombocytopenia in the newborn has been shown [4–6]. The alloimmunization is strongly associated with the *HLA-DRB3*01:01* allele [3, 7, 8]; however, only about 30% of the women with this HLA antigen are immunized. Except for the incompatibility in platelet antigen and the association to HLA, other factors which may influence the immune response to HPA-1a have not been identified.

In the present study, we have examined the maternal ABO blood groups and frequency of HPA-1a-immunization of the women identified in the large prospective screening and intervention study carried out in Norway from 1995 to 2004. We included 152 HPA-1a-immunized women, 146 of whom had altogether 158 HPA-1-incompatible pregnancies in the screening study. The ABO distribution among

immunized women was investigated, and the maternal ABO phenotype and *ABO* genotype was correlated to the severity of thrombocytopenia of the newborn.

2. Materials and Methods

2.1. Patients. Pregnant women were recruited for HPA-1 allotyping from three regions in Norway between December, 1995 and March, 2004 [3]. Samples for routine Rh(D) typing were also used for determining HPA-1 allotype by flow cytometry (anti-CD61 mAb), enzyme-linked immunosorbent assay (ELISA), or polymerase chain reaction (PCR) as previously described [9]. A total of 100,448 pregnant women were typed for the platelet antigen HPA-1a, and 2,111 of those were HPA-1a negative (2.1%). Of these, 1,990 were further tested, and anti-HPA-1a antibodies were detected in 154 women during the pregnancy. In total, 146 of these immunized women underwent 158 HPA-1a-incompatible pregnancies. ABO blood group typing was performed by conventional technique. Genomic typing of HPA-1 (*ITGB3;* rs5918 in dbSNP) and *ABO* in the neonates was performed in samples from cord blood or buccal swabs. For the newborns, the *ABO* genotype was used to predict the ABO blood group. In this context, we have defined ABO incompatibility only as an A_1 phenotype in the newborn, in blood group O mothers, because individuals with A_2, and the majority of individuals with B phenotype, express only low levels of corresponding antigens on the surface of platelets [2, 10–12]. Thrombocytopenia was defined as a platelet count $\leq 150 \times 10^9$/L, and severe thrombocytopenia less than 50×10^9/L measured in cord blood and/or capillary blood at birth. Detection of anti-HPA-1a IgG antibodies was performed by flow cytometry and quantified with monoclonal antibody immobilization of platelet antigen assay (MAIPA) [3], by using the anti-CD61 monoclonal antibody clone Y2/51 (Dako, Glostrup, Denmark) for immobilisation of platelet glycoproteins. Women were tested at several time points during the pregnancy, and those with a positive antibody test at any time during the pregnancy were characterized as immunized. Nineteen women were primary immunized during the studied pregnancy, 13 of these were primigravida. All others may have been immunized in connection with a prior pregnancy. Prior affected pregnancies were not excluded as a cause of severe NAIT. The NAIT diagnosis was based on maternal anti-HPA-1a antibodies and HPA-1a antigen incompatibility. Other possible reasons for thrombocytopenia (infection, maternal ITP, etc.) were not registered. Informed consent was provided in accordance with the declaration of Helsinki. The study was approved by the Regional Committee for Medical Research Ethics, North Norway (approval no. P-REK V 13/1995).

2.2. ABO Genotyping. ABO genotyping was performed by PCR-RFLP analysis to detect six major alleles, A^1, A^2, B, O^1/O^{1v}, and O^2 (also known as *A101/A201/B101/O01/O02/O03*) according to the nomenclature used by the Blood Group Antigen Gene Mutation Database, dbRBC [13], and further discrimination between the common O^1 and O^{1v} alleles [*O01/O02*] was performed using primers and reaction conditions as described by Olsson and Chester [14, 15] with some modifications: HotStarTaq polymerase 5 U/μL (QIAGEN, Hilden, Germany) was used with the following cycling programs for both analyses: 95°C 15 min, 10 cycles of 94°C for 10 seconds, 63°C for 30 seconds and 72°C for 30 seconds followed by 25 cycles of 94°C for 10 seconds, 61°C for 30 seconds and 72°C for 30 seconds for samples with 100 ng DNA template. For samples with ~25 ng template and <10 ng DNA template, 1 and 2 extra cycles at each of the annealing temperatures was performed, respectively. Subsequently, digestion with endonucleases and qualitative analyses of product were performed [14, 15]. Ambiguous results were confirmed with selected primer sets from a recently published PCR-ASP method for *ABO* genotyping [16]. For simplicity, only one terminology, dbRBC, will be used throughout this paper. Genotypes are written as *X/X*.

2.3. Statistical Analysis. Standard statistical calculations as mean, relative risk, Chi-square test, analysis of variance with Bonferroni's test as post hoc analysis, and plots were performed with computer software SPSS for windows (Statistical Package for the Social Sciences, Version 16.0 SPSS Inc., Chicago, Ill, USA). $P < 0.05$ was considered significant.

3. Results

3.1. The ABO Phenotype Distribution of the Immunized Mothers. The ABO phenotype distribution among 154 HPA-1a immunized women was similar to the distribution of the general Norwegian population [17] adjusted to statistics for 2005 (data not shown), indicating that the maternal ABO type does not influence the risk of HPA-1a immunization.

3.2. Maternal ABO Blood Group and Risk of Severe NAIT. In 158 HPA-1-incompatible pregnancies with 83 cases of NAIT, there were 54 cases of severe NAIT. The maternal ABO phenotypes were compared to the platelet count in their neonates (Table 1); 46.6% of the immunized women with blood group A gave birth to children with severe NAIT, compared to 20.0% among the immunized mothers with blood group O. The relative risk of NAIT (platelet count $\leq 150 \times 10^9$/L) in the neonates of HPA-1a immunized women with blood group O as compared to blood group A was 0.67 (95% CI 0.48–0.94), whereas the relative risk of severe NAIT (platelet count $<50 \times 10^9$/L) in the neonates of HPA-1a immunized women with blood group O was 0.43 (95% CI 0.25–0.75) as compared to the neonates of women with blood group A. However, the frequency of moderate NAIT (platelet count 50–150×10^9/L) was not lower among the blood group O mothers. 91% of the immunized women carried the *HLA-DRB3*01 : 01* allele. There were no cases of severe NAIT among *HLA-DRB3*01 : 01*-negative mothers.

3.3. ABO Incompatibilities between Mothers and Newborns. To investigate whether the ABO incompatibility between mother and foetus could explain the difference in the frequency of severe NAIT, newborns were *ABO* genotyped as basis for the prediction of their ABO phenotype. One hundred and thirty out of 146 newborns were *ABO* genotyped

Table 1: The maternal ABO type distribution in the pregnancies compared to the severity of NAIT.

Maternal ABO type	Numbers of newborns with platelet count $<50 \times 10^9$/L (% within ABO type; 95% CI)	Numbers of newborns with platelet count 50–150 $\times 10^9$/L	Numbers of newborns with platelet count $>150 \times 10^9$/L	P value†
A	34 (46.6; 0.36–0.58)	13	26	0.005
O	12 (20.0; 0.12–0.32)	14	34	
B	7 (38.9; 0.20–0.61)	1	10	
AB	1 (14.3; 0.03–0.51)	1	5	
Total	54	29	75	

Relative risk of NAIT was 0.67 (95% CI 0.48–0.94) in neonates born of women with blood group O versus blood group A.
Relative risk of severe NAIT was 0.43 (95% CI 0.25–0.75) in neonates born of women with blood group O versus blood group A.
†Chi-square test (two-sided) for frequencies of NAIT and severe NAIT in blood group O compared to blood group A.

(restricted by lack of material). For ABO-incompatibility studies, thus only 52 of 60 blood group O mother-child pairings could be included. The fifty-two mothers with blood group O gave birth to 16 A-incompatible (blood group A_1) and 36 compatible (sixteen blood group O, one B, and five A_2) children. Four of the 16 A-incompatible pregnancies resulted in a newborn with severe thrombocytopenia compared to 6 of the 36 ABO-compatible pregnancies. This indicates that ABO incompatibility is not the underlying cause of the observed phenomenon reported in the present study.

3.4. The ABO Genotype of the Mothers and Platelet Counts in the Newborn. The *ABO* genotype of 143 HPA-1a-immunized women who gave birth to 155 HPA-1a-positive neonates was determined (data not shown). The overall *O* allele frequencies among the immunized women were *O01* 0.56, *O02* 0.42, and *O03* 0.02. Individuals with blood groups A, B, and O were further subgrouped based on genotyping, and thus, the frequencies of newborns with severe NAIT within each subgroup were compared. The cases with maternal blood group AB were excluded for further analysis due to the low number of individuals. Analysis of the platelet counts in newborns of mothers with different *ABO* genotypes revealed that the frequency of newborns with severe NAIT differed (Pearson Chi-square $P = 0.0036$) among the maternal *ABO* genotype groups.

Among blood group A mothers, the frequency of newborns with severe NAIT was 42% in pregnancies where the mother carried only one *A* allele (*A101* or *A201*), compared to 69% where mothers carried two *A* alleles (relative risk 0.61; 95% CI 0.38–0.98). In pregnancies where the mother had blood group O, the frequency of newborns with severe NAIT was 9%, where the mother did not carry any *O02* allele, compared to 27% where the mother carried one or two *O02* alleles; however, this did not reach statistical significance (relative risk 0.33 NS $P = 0.13$).

Platelet counts in newborns of mothers with blood group A and O are plotted in Figure 1. The mean antibody levels between these groups were not significantly different: 11.6 IU/mL for blood group A mothers, 1.8 IU/mL for O02-negative blood group O mothers, and 11.1 IU/mL for O02-positive blood group O mothers ($P = 0.18$ one-way ANOVA). However, the correlation between the maternal antibody level and platelet count in the newborn for these cases was described in Killie et al. [6].

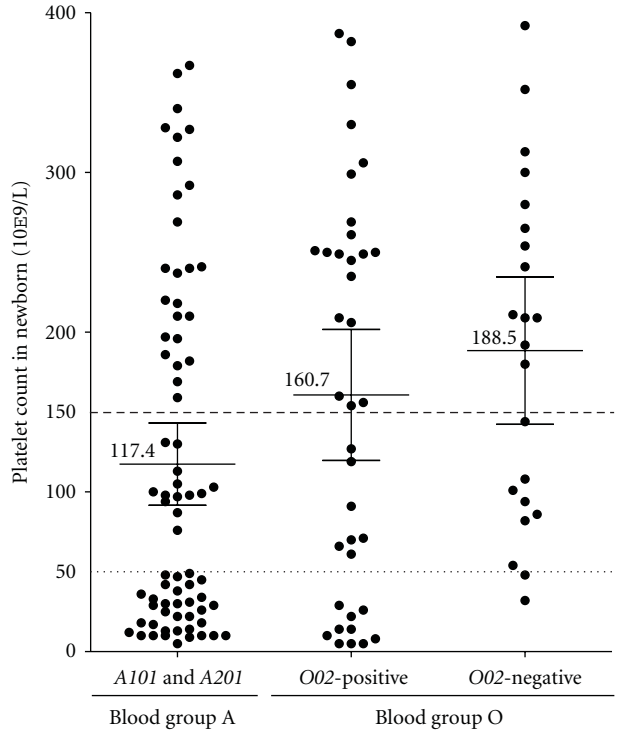

Figure 1: The platelet count at delivery in HPA-1a-positive newborns was grouped according to maternal ABO type (A or O). Platelet count $\leq 150 \times 10^9$/L (dashed line) is defined as NAIT and $<50 \times 10^9$/L (dotted line) as severe NAIT. The mean platelet counts in the three groups are indicated, with error bars representing 95% CI. Mean platelet counts are significantly different ($P < 0.019$, one-way ANOVA). The mean antibody levels between these groups are not significantly different: 11.6 IU/mL for blood group A mothers, 11.1 IU/mL for *O02*-positive blood group O mothers, and 1.8 IU/mL *O02*-negative blood group O mothers.

Among the NAIT cases, defined as platelet count $\leq 150 \times 10^9$/L, the mean platelet count in newborns of homozygous *O01/O01* mothers was higher (83.2×10^9/L) than in the newborns with *O02*-positive mothers with blood group O (43.7×10^9/L) or in newborns of women with blood group A (46.1×10^9/L) (Table 2). Together, these data support

Table 2: Platelet counts in the newborns with NAIT.

Maternal ABO types		N^*	Median platelet count	Mean platelet count (95% CI)	P value with Bonferroni correction
Blood group O	Genotype *O01/O01*	9	86	83.2 (56.9–109.6)	
	Genotypes *O01/O02* and *O02/O02*	17	21	43.7 (22.7–64.7)	0.043†
Blood group A		47	31	46.1 (35.0–57.2)	0.028†

*Total number of pregnancies resulting in a newborn with NAIT (platelet count ≤150) was 83. DNA for genotyping was available for 68 women with blood group O or A, and they had altogether 73 HPA-1-incompatible pregnancies.
†Comparison with platelet counts in children born of women with the genotype *O01/O01*.

our hypothesis that there are genetic properties among the immunized women influencing the risk of severe NAIT in the newborn.

4. Discussion

The ABO phenotype distribution among the HPA-1a immunized women is similar to the distribution in the Norwegian population, indicating that the generation of an immune response with antibody synthesis is independent of the ABO blood group of the mother. However, we observed that whereas only 20% of pregnancies among the immunized women with blood group O resulted in severe NAIT in the newborn, 47% of the immunized women with blood group A had newborns with severe thrombocytopenia. A recent retrospective study by Bertrand et al. did not find any significant correlation between the severity of the thrombocytopenia and the *ABO* genotype [18]. As these authors propose, the discrepancy between Bertrands's and our study may be due the retrospective/prospective nature of the studies. We found no indications that the low frequency of severe NAIT in the children of women with blood group O was due to ABO incompatibility between mother and foetus. Additional measurements of maternal anti-RBC IgG antibody in the women with blood group O could have given further information of any influence of potential antibodies directed against the A antigen carried by αIIb on platelets. Another hypothesis that could explain the lower frequency of newborns with severe NAIT among the immunized mothers with blood group O, compared to blood group A, is that the *ABO* gene is located close to a gene encoding an immunoregulatory factor with polymorphic variants. In order to approach this question, we compared NAIT to *ABO* genotypes. The allelic differences in the gene encoding the A/B glycosyltransferases are defined by SNPs that changes the amino acid sequence of the enzyme and thereby its glycosylating properties. The *A101*, *A201*, *B101*, *O01*, *O02*, and *O03* alleles all produce transcripts (although *A* transcripts are virtually undetectable in peripheral blood) [19, 20], but the *O01* and *O02* transcripts both contain a shift in the reading frames that will severely truncate any resulting protein and leave it without enzymatic activity [21]. It is still unclear if these short nonfunctional proteins are expressed at all although it has been suggested [22].

The *ABO* genotype frequencies in the Norwegian population are not known, but the *O* allele frequencies observed in immunized women are similar to the frequencies reported for a Swedish population [15], where the *O02* constitutes about 40% of all *O* alleles and *O03* allele is infrequent. This further shows that the generation of an immune response to HPA-1a is independent of ABO blood groups. However, when it comes to development of NAIT, the different risks of severe thrombocytopenia observed in genetic subgroups of blood group A support the hypothesis that a genetic linkage may be involved, rather than the ABO phenotype itself even though the mechanism is still not understood. Although the differences in the *O02*-positive and *O02*-negative subgroups of blood group O do not reach statistical significance, an interesting trend is observed.

Phylogenetic analyses of the *ABO* locus have shown that the *O02* probably is an ancient allelic lineage at the *ABO* locus, separate from the *A101* and *O01* alleles [23]. Therefore, it is interesting to subdivide the blood group O women according to their genotype. The 9q34 chromosomal region, where the *ABO* gene is located [24], contains several loci encoding immune response regulating genes. There is obviously no genetic linkage between the *ABO* [9q34] and *ITGB3* [17q21] loci. The association of the ABO type to the development of severe NAIT could be due to a potential linkage to one or more gene(s) encoding regulatory factors. Further investigation has to be conducted to find out whether such factors are linked to the *ABO* locus in a way that can explain our observation.

5. Conclusions

The development of severe NAIT in newborns is caused by transfer of platelet-reactive antibodies during pregnancy; however, several biological factors likely play a role in the immune response mechanism. In the present study, with data from a prospective NAIT study, we showed that the risk of severe NAIT due to anti-HPA-1a antibodies is correlated to maternal ABO types. The results indicate that there are genetic properties related to the maternal *ABO* genotype that influence the immune response that cause severe thrombocytopenia in the newborn of anti-HPA-1a immunized mothers.

Authors' Contribution

B. Skogen was responsible for conception of the study. M. T. Ahlen contributed to study design, performed the experiments, collected data, and performed statistical analyses. A. Husebekk supervised the research; M. L. Olsson

contributed with study design and interpretation of *ABO* analyses; J. Kjeldsen-Kragh and M. K. Killie contributed to study design and interpretation of data, the paper was written by M. T. Ahlen and B. Skogen, with contributions from A. Husebekk, J. Kjeldsen-Kragh, M. K. Killie, and M. L. Olsson. All authors critically reviewed the paper and approved the paper for publication.

Conflicts of Interests

The authors reported no potential conflicts of interest.

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Research Article

Mapping of Minimal Motifs of B-Cell Epitopes on Human Zona Pellucida Glycoprotein-3

Wan-Xiang Xu,[1] Ya-Ping He,[1] Jian Wang,[1] Hai-Ping Tang,[1] Hui-Juan Shi,[1] Xiao-Xi Sun,[2] Chao-Neng Ji,[3] Shao-Hua Gu,[3] and Yi Xie[3]

[1] *Department of Reproductive Biology, Shanghai Institute of Planned Parenthood Research, Shanghai 200032, China*
[2] *Shanghai Jiai Genetic & IVF Center, Obstetrics and Gynecology Hospital, Shanghai Medical College, Fudan University, Shanghai 200011, China*
[3] *State Key Laboratory of Genetic Engineering, Institute of Genetics, School of Life Science, Fudan University, Shanghai 200433, China*

Correspondence should be addressed to Wan-Xiang Xu, xuwx@sippr.stc.sh.cn and Yi Xie, yxie@fudan.edu.cn

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The human zona pellucida glycoprotein-3 (hZP3) by virtue of its critical role during fertilization has been proposed as a promising candidate antigen to develop a contraceptive vaccine. In this direction, it is imperative to map minimal motifs of the B cell epitopes (BCEs) so as to avoid ZP-specific oophoritogenic T cell epitopes (TCEs) in the ZP3-based immunogens. In this study, based on known results of mapping marmoset and bonnet monkey ZP3 (mstZP3 and bmZP3), two predictable epitopes$^{23–30 \text{ and } 301–320}$ on hZP3 were first confirmed and five minimal motifs within four epitopes on hZP3 were defined using serum to recombinant hZP3a$^{22–176}$ or hZP3b$^{177–348}$ as well as a biosynthetic peptide strategy. These defined minimal motifs were QPLWLL$^{23–28}$ for hZP3$^{23–30}$, MQVTDD$^{103–108}$ for hZP3$^{93–110}$, EENW$^{178–181}$ for hZP3$^{172–190}$, as well as SNSWF$^{306–310}$ and EGP$^{313–315}$ for hZP3$^{301–320}$, respectively. Furthermore, the antigenicity of two peptides for hZP3$^{172–187}$ and hZP3$^{301–315}$ and specificity of the antibody response to these peptides were also evaluated, which produced high-titer antibodies in immunized animals that were capable of reacting to ZP on human oocytes, r-hZP3b$^{177–348}$ protein, as well as r-hZP3$^{172–190}$, r-hZP3$^{303–310}$, and r-hZP3$^{313–320}$ epitope peptides fused with truncated GST188 protein.

1. Introduction

The human zona pellucida (hZP) is an extracellular matrix surrounding eggs, which consists of four sulfated glycoproteins designated as ZP1, ZP2, ZP3, and ZP4 [1, 2]. ZP glycoproteins mediate several critical events during fertilization process such as initial recognition and binding of the spermatozoa to the egg in a species-specific manner, induction of the acrosome reaction in the zona-bound spermatozoon, and prevention of polyspermy [3, 4]. By virtue of their critical role during fertilization, ZP glycoproteins have been proposed as target for developing contraceptive vaccines [5, 6]. Although active immunization with purified or recombinant ZP (r-ZP) proteins induces infertility in various mammalian species, the contraceptive efficacy is invariably associated with either transient alteration or complete loss of ovarian function [7–10]. The observed ovarian pathology following active immunization with ZP antigens may be due to (i) presence of ZP-specific T-cell epitopes (TCE) within a B-cell epitope (BCE), for example peptide corresponding to mouse ZP3 (mZP3) amino acid (aa) residues 330–342 (peptide$^{330–342}$) [11], (ii) autoimmune ovarian disease (AOD) that can be adoptively transferred by the ZP3 peptide-activated CD4$^+$ T-cells to naïve recipients but does not occur when only antipeptide antibodies are administered [12], and (iii) the minimal and modified BCE peptide$^{335–342}$ of mZP3, which had no longer a ZP-specific TCE (phenylalanine, a key residue of the "oophoritogenic" TCE, substituted by alanine), induced infertility in mice with eight different haplotypes without any AOD when it was co-linearly synthesized with a foreign "promiscuous" TCE [13].

The above observations clearly suggest a new possible approach of developing BCE peptide-based ZP vaccines for fertility control, which should be devoid of any ZP-specific TCE.

Therefore, efforts have been made by several laboratories to delineate infertility-associated BCEs of ZP proteins since a BCE on mZP3 was first identified by Dean et al. [14]. For instance, five BCE peptides$^{45–64,\ 93–110,\ 137–150,\ 172–190\ \text{and}\ 334–341}$ of hZP3 have been mapped [15–17] and additional two epitopes corresponding to aa residue 23–30 and 301–320 of hZP3 have also been predicted based on the mapping results of marmoset ZP3 (mstZP3) and bonnet monkey (Macaca radiata; bmZP3) proteins [18, 19], because their aa sequences are highly conserved among mstZP3 and hZP3 as well as bmZP3 and hZP3 proteins. To eliminate potential oophoritis-inducing TCE within a mapped longer BCE peptide, the identification of minimal motif of mapped BCEs on hZP3 has been hampered due to the limitation of available mapping methods. So far, only the minimal motif of the BCE on the C-terminus of hZP3 has been identified using rabbit serum [16].

We have reported previously the identification of a minimal motif of hZP4$^{314–319}$ epitope using improved peptide biosynthesis strategy for the first time, where the truncated streptavidin (Stv108) and glutathione S-transferase (GST188) were used as protein carrier, respectively [20]. In addition, the sera against r-hZP3$^{22–176}$ (hZP3a) and r-hZP3$^{177–348}$ (hZP3b) were made, which reacted with human oocyte [21]. Therefore, the main aim of the present study was to map minimal motifs of four linear BCEs on hZP3 with biosynthetic peptides and antibodies against r-hZP3a or r-hZP3b. Moreover, the immunogenicity of two chemically synthesized peptides$^{171–186\ \text{and}\ 301–315}$ of hZP3, conjugated to keyhole limpet hemocyanin (KLH), has also been evaluated in rabbits.

2. Materials and Methods

2.1. Plasmids, Antibodies, and Peptides. The plasmids pXXStv-3 and pXXGST-1 were used to express various biosynthetic peptides in *E. coli* [20]. Rabbit sera against r-hZP3a and r-hZP3b prepared as described previously were used for BCE identification and minimal motif mapping [21]. Peptides FSLRLMEENWNAEKRS (P1) and SFSKPSNSWFPVEGP (P2) corresponding to hZP3$^{172–187}$ and hZP3$^{301–315}$ as well as PETQPGPLTLELQIAKDK (P3) corresponding to hZP4$^{308–325}$ were produced on an APEX396 synthesizer by Sangon Co. (Shanghai, China), with more than 90% purity on HPLC. The peptides P1 and P2 were used as antigens to test antibody against P1 or P2 by ELISA, and peptide P3 was used as unrelated control peptide in ELISA.

2.2. Other Reagents. All chemicals were purchased from Sangon Co. unless otherwise stated.

2.3. Biosynthesis of 6/8mer–20mer Peptides. A set of biosynthetic peptides (numbering P4–P11) corresponding to P4 (QPLWLLQG), P5 (ECQEATLMVMVSKDLPGTGK), P6 (EVGLHECGNSMQVTDDAL), P7 (PIECRYPRQGNVSS), P8 (FSLRLMEENW), P9 (FSLRLMEENWNAEKRSPTF), P10 (SFSKPSNSWFPVEGPADICD), and P11 (RRQPHVMS) of hZP3$^{23–30,\ 45–64,\ 93–110,\ 137–150,\ 172–181,\ 172–190,\ 301–320\ \text{and}\ 334–341}$ were expressed in *E. coli* as truncated GST188 or Stv108 fusion proteins as described earlier [20]. Similarly, four sets of 35 overlapping 6–8mer peptides (P12–P46) corresponding to P4, P6, P9, and P10 sequences were also made, which overlapped each other by 5–7 residues.

Briefly, the synthesized annealed DNA fragments encoding each P4 to P46 peptides corresponding to the hZP3 cDNA sequence [22], incorporating BamH I and TAA-Sal I cohesive end on their 5′ and 3′ ends, were inserted into the BamH I and Sal I sites downstream of the Stv108 or GST188 gene in pXXStv-3 or pXXGST-1 plasmid. The resultant recombinant plasmids expressing each target short peptide fused with Stv108 or GST188 protein were transformed into the BL21(DE3)(pLysS) *E. coli* strain (Novagen, Inc., Madison, Wis, USA). Each recombinant clone was first grown in 3 mL of Luria Broth (LB) containing 100 μg mL^{-1} ampicillin at 30°C with continuous shaking at 200 rpm overnight. Next day, 60 μL of cell suspension was inoculated in 3 mL of fresh LB and grown until the cell density reached 0.6–0.8 at OD600, and then further grown for 4 h at 42°C to induce expression of the recombinant protein. For screening positive recombinant clones, each cell pellet harvested from induced target clones was first used to run SDS-PAGE gel using the pellet containing Stv108 or GST188 protein expressed by pXXStv-4 or pXXGST-2 plasmid as negative controls, and nucleotide (nt) sequence of all the recombinant clones were subsequently determined (United Gene Holding Ltd., Shanghai). The cell pellets containing each short peptide fusion protein were stored at −20°C.

2.4. SDS-PAGE and Western Blotting. Cell pellets obtained from 3 mL culture were boiled in 400 μL of 1x sample loading buffer for 5 min and proteins were resolved by SDS-PAGE under reducing conditions using 15% gels [23]. Gels were either stained with Coomassie brilliant blue G-250 for analyzing the bands of fusion proteins or processed for Western blot by electrotransferring the proteins onto 0.2 μm nitrocellulose membrane (Whatman GmbH, Dossel, Germany) [24]. Complete transfer of proteins was ensured by staining the nitrocellulose membrane with 0.1% (w/v) Ponceau S. Nitrocellulose membrane was subsequently processed for Western blotting using rabbit antisera against r-hZP3a and r-hZP3b (1 : 300 dilution in PBS containing 0.05% Tween 20 and 1% skim milk powder) or immune sera against P1 or P2 (1 : 2000 dilution). Specific antigen-antibody reactions on the membrane were visualized by using goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (Proteintech Group, Inc., Chicago, Ill, USA) at 1 : 1000 dilution. The blot was developed by using 3,3′-diaminobenzidine (DAB) (Sigma, Mo, USA) in 50 mM PBS containing 0.05% H_2O_2. The reaction was stopped by washing the membrane extensively with MQ water. In some cases, enhanced chemiluminescence was also performed using ECL plus Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions.

2.5. Immunization of Animals. Care and treatment of the animals was based on the standard laboratory animal care

protocols approved by the Institutional Animal Care Committee. Six male New Zealand White rabbits were obtained from SIPPR-BK Lab Animal Co., Ltd. (Shanghai, China). The immunization schedule was as follows: six rabbits (number 073–076) weighing 2.0 ± 0.5 kg were immunized intramuscularly with 0.5 mg of chemically synthesized peptide P1 or P2 conjugated with KLH (Sigma) and emulsified in complete Freund's adjuvant (CFA) (Sigma) at multiple sites on the rabbit's back, respectively. The animals were boosted three times intramuscularly with 0.25 mg of same peptide antigen emulsified in incomplete Freund's adjuvant (Sigma) at 2 week intervals. Serum samples from immunized animals were collected 7 days after the third booster, and the antibody titer was assessed by ELISA. Nonimmune sera from two animals (number 077-078) who only received CFA and P3 as unrelated peptide antigen were used as negative controls in the ELISA, respectively.

2.6. ELISA and Immunofluorescence

2.6.1. ELISA. The 96-well ELISA plates (Greiner bio-one; Germany) were coated with 50 μL of synthetic peptide P1 or P2 (100 ng per well) overnight at RT. Unbound synthetic peptide was washed off with PBS containing 0.05% Tween 20 (PBST), and sera against P1 or P2 diluted 1 : 50 in blocking buffer (0.01 M PBS containing 5% skim milk powder and 0.05% Tween) were added to the wells (50 μL/well) at twofold serial dilutions. After incubation for 2 h at RT, plates were washed three times with PBST. To visualize specific peptide-antibody reactions, 50 μL of goat-anti-rabbit IgG conjugated to HRP diluted 1 : 1,000 in PBS was added to wells and incubated for 1 h at RT. All wells were treated with 0.4 mg mL^{-1} o-phenylenediamine and 0.015% (v/v) H_2O_2 after washing as before and the reaction was stopped with H_2SO_4. Finally, the absorbance was read at 490 nm, according to the manufacturer's instructions (Thermo Fisher Scientific, Pittsburgh, Pa, USA, Product number 34062T) using an ELX 800 Universal Microplate Reader (Bio-TEK Instruments, Inc. Vt, USA). For negative control, the same amount of unrelated P3 synthetic peptide was used for coating the wells. In addition, rabbit preimmune serum was also used as negative control

2.6.2. Immunofluorescence. Experiments using human oocytes was approved by the Institutional Ethics Committee. Human oocytes that had failed to fertilize during in vitro fertilization (IVF) treatments were kindly donated, and a signed written consent for use of oocytes was obtained from all participants. Reactivity of antisera against P1 or P2 with native human ZP was evaluated by an indirect immunofluorescence. Briefly, all oocytes were washed thrice with PBS containing 1 mg mL^{-1} of polyvinylalcohol (PVA), and then fixed in 4% (w/v) paraformaldehyde in 50 μL of PBS for 5 min at RT. Next, the fixed oocytes were washed with PBS-PVA and incubated for 45 min in a blocking solution containing 3% normal goat serum. Further, oocytes were incubated with 1 : 50 dilution of preimmune or immune sera in PBS-PVA for 1 h at 37°C after washing with PBS-PVA. Finally, oocytes were treated with 1 : 500 dilution of Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, Calif, USA) for 30 min at 37°C, and after washing, the treated oocytes in PBS-PVA were examined under a Nikon TE300 inverted microscope (Nikon Co., Tokyo, Japan).

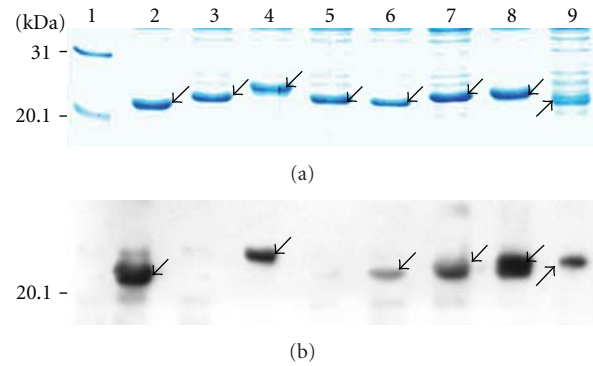

Figure 1: (a) SDS-PAGE analysis of expressed P4–P11 peptides fused with GST188 protein. (b) Western blotting of each BCE peptide using a mixture of sera to r-hZP3a and r-hZP3b. Lane 1, prestained protein marker; 2, lanes 2–9, P4–P11 fusion proteins. Arrows in a-b indicate the bands comprising expressed P4–P11 fusion proteins and their respective bands.

3. Results

3.1. Reidentification of Known and Predictable Linear BCEs on hZP3 Protein. To determine whether those identified (hZP3$^{45–64,\ 93–110,\ 137–150,\ 172–190\ \text{and}\ 334–341}$) and predicted (hZP3$^{23–30,\ 172–181\ \text{and}\ 301–320}$) BCE peptides could be recognized by rabbit sera to r-hZP3a and r-hZP3b, the P4–P11 peptides, which were fused with truncated GST188, were constructed with DNA recombinant technology. As shown in Figure 1(a), P4–P11 fusion proteins were expressed in *E. coli*. Surprisingly, the electrophoretic mobilities of some short peptides fused with truncated GST188 did not seem to match their molecular weight, such as, 20mer P5 protein (Figure 1(a), Lane 3) migrated similarly to that of 14mer P7 protein (Lane 5) than to that of 18mer P6 protein (Lane 4) and so on. Although this aberrant behavior cannot be explained at present, these electrophoretic results should be reliable as their respective nt sequence matched with the aa sequence. Further, expression of the respective fusion protein was not observed in the uninduced clones (data not shown).

Six peptides (P4, P6, P8, P9, P10, and P11) of hZP3 could be recognized by antiserum against r-hZP3a or r-hZP3b, but P5 and P7 failed to react with both the immune sera in Western blot (Figure 1(b)). The results confirmed three BCE peptides (P6, P9, and P11) of hZP3 out of five BCE peptides identified previously. All three predicted BCE peptides also reacted with antibodies against r-hZPa/r-hZP3b. In a word, this study showed that there at least were five BCE peptides on hZP3 protein.

3.2. Mapping of Minimal Motifs on P4, P6, P9, and P10 Epitope Peptides. Based on initial mapping result, the peptides P4,

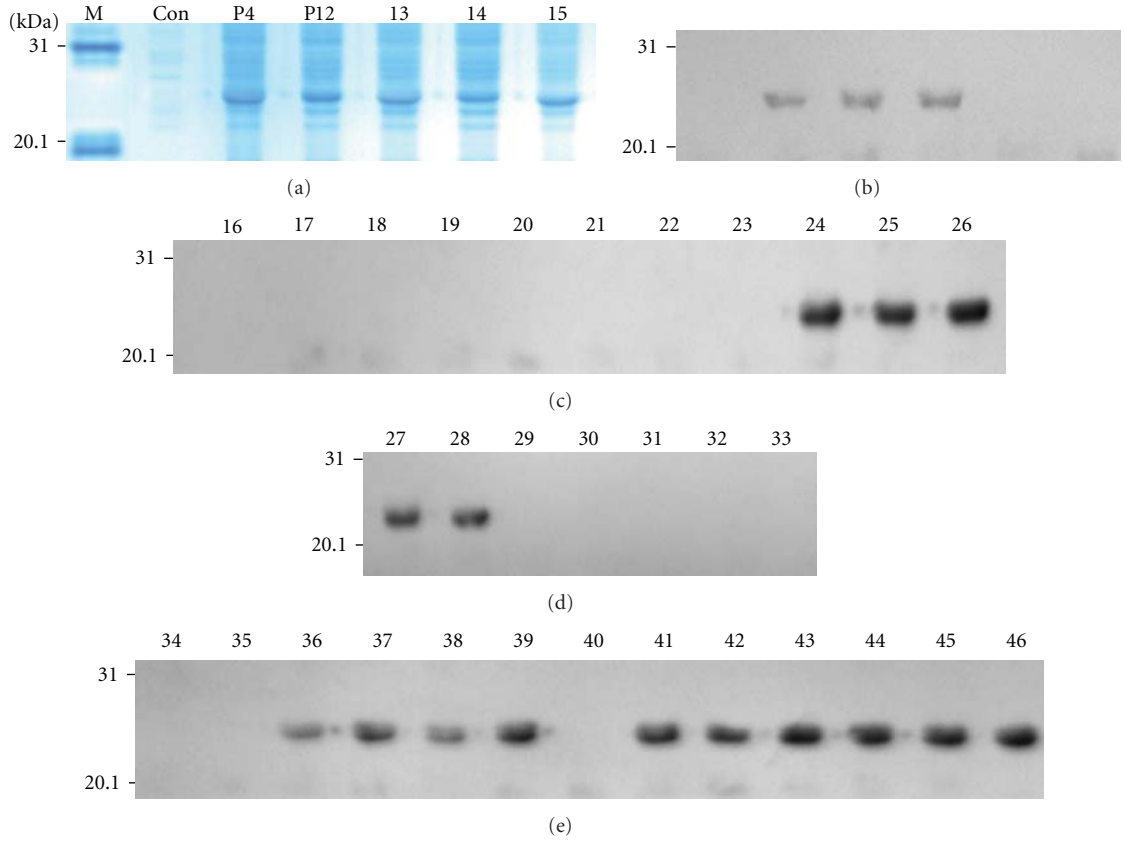

Figure 2: (a) SDS-PAGE analysis of expressed P4 and P12–P15 fusion proteins. (b)–(e) Western blotting of GST188-8mer peptides from P4, P6, P9, and P10 peptides detected using sera to r-hZP3a or r-hZP3b. Note: M, prestained protein marker; Con, uninduced total cell proteins as negative control; P4 and P12–46, total cell proteins of expressed P4; P12–P46 8mer peptide fusion proteins.

P6, P9, and P10 were selected to identify their minimal motifs with sera to r-hZP3a or r-hZP3b, except P11 that its motif has been known [16]. Four sets of thirty-seven 6–8mer peptides with an overlap of 5–7 aa residues for P4, P6, P9, and P10 peptide were constructed and used to map minimal motifs in this study. All P4 and P12 to P46 peptides were expressed in *E. coli* at a higher level (Figure 2(a), others not shown). As shown in Figure 2, three (b-c), two (d), as well as four and six (e) overlapping 6–8mer peptides fused with GST188 were recognized by serum against r-hZP3a or r-hZP3b. These 36 constructs shared sequences of 6 aa (P4 and P6), 7 aa (P9), as well as 5 aa and 3 aa (P10), respectively (Figure 3). Thus, their minimal binding motifs were localized to residues $QPLWLL^{23–28}$, $MQVTDD^{103–108}$, $EENWNAE^{178–184}$, $SNSWF^{306–310}$, and $EGP^{313–315}$ on the epitope peptides P4, P6, P9, and P10, of which two minimal motifs on peptide P10 were identified, suggesting that there were two nested BCEs on it.

Additionally, we prejudged that the peptide P8 of $hZP3^{172–181}$ should be recognized by serum to r-hZP3b, because the mapped epitope $sequence^{171–180}$ of mZP3 [25] is 100% conserved among mZP3 and hZP3 at the amino acid level. However, they did not generate a blotted brown band at the position of Stv108-P8 fusion protein when initially using DAB coloration due to its relatively lower sensitivity (result not shown). Therefore, a set of P4–P11 peptides fused with GST188 was again constructed, which were suited to chemoluminescence detection on the blotted membrane and then identified when using high-sensitivity ECL plus Western blotting detection reagents. As a result, the peptides P8 and P9 were recognized by serum to r-hZP3b (Figure 1(b), lanes 6-7). Thus, the minimal $motif^{178–181}$ of peptide P9 was finally defined according to the shared sequences of 4 aa residues (EENW) between P8 and the minimal motif of $hZP3^{178–184}$ mapped initially.

3.3. Antigenicity of Peptides P1 and P2. To investigate and compare antigenicity of peptides P1 and P2, each two rabbits (number 073-074 and number 075-076) were immunized with synthetic peptide P1 or P2 antigen in FCA, which were conjugated with KLH. As shown in Figure 4, each peptide antigen all elicited higher antibody responses against peptide P1 or P2 in immunized animals, of which the rabbit (number 073) immunized with P1 antigen showed antibody titers of 6.4×10^4, whereas that level in the rabbit (number 076) immunized with P2 antigen reached approximately 5.1×10^5 in ELISA assay.

3.4. Specificity of Antibodies against Peptides P1 and P2. The rabbit sera against P1 or P2 reacted to not only synthetic peptide P1 or P2 in ELISA, but also to P9 ($hZP3^{172–190}$) or

The position and blotting results of 8mer peptide of hZP

Peptide number	Amino acids	Position in hZP3	Peptide number	Amino acids	Position in hZP3
P4	**QPLWLL**QG	23–30	P27	M**EENWNAE**	177–184
P12	**QPLWLL**Q	23–29	P28	**EENWNAE**K	178–185
P13	**QPLWLL**	23–28	P29	ENWNAEKR	179–186
P14	PLWLLQ	24–29	P30	NWNAEKRS	180–187
P15	LWLLQG	25–30	P31	WNAEKRSP	181–188
			P32	NAEKRSPT	182–189
P16	EVGLHECG	93–100	P33	AEKRSPTF	183–190
P17	VGLHECGN	94–101			
P18	GLHECGNS	95–102	P34	SFSKPSNS	301–308
P19	LHECGNSM	96–103	P35	FSKPSNSW	302–309
P20	HECGNSMQ	97–104	P36	SKP**SNSWF**	303–310
P21	ECGNSMQV	98–105	P37	KP**SNSWF**P	304–311
P22	CGNSMQVT	99–106	P38	P**SNSWF**PV	305–312
P23	GNSMQVTD	100–107	P39	**SNSWF**PVE	306–313
P24	NS**MQVTDD**	101–108	P40	NSWFPVEG	307–314
P25	S**MQVTDD**A	102–109	P41	SWFPV**EGP**	308–315
P26	**MQVTDD**AL	103–110	P42	WFPV**EGP**A	309–316
			P43	FPV**EGP**AD	310–317
			P44	PV**EGP**ADI	311–318
			P45	V**EGP**ADIC	312–319
			P46	**EGP**ADICQ	313–320

Figure 3: The synthetic 6/8mer peptide sequences from hZP3 protein. The **green** highlight indicates the common sequence recognized by antibodies to r-hZP3a or r-hZP3b in P4, P6, P9, and P10 fusion proteins.

P10 ($hZP3^{301–320}$)-GST188 fusion protein (Figures 5(a) and 5(b), Lane 2 and 7) as well as native human ZP in indirect immunofluorescence (Figures 5(i) and 5(j)). As shown in Figure 5(a), the serum against P1 did not react with Stv108 and GST188 carrier proteins (Lanes 5-6), so does the serum against P2 (data not shown), suggesting there was no cross-reactive antibodies with Stv108 and GST188 proteins in their immune sera. In addition, the serum to P2 reacted to 8mer peptides P37 and P44 containing a mapped minimal motif SNFWF or EGP (Figure 5(b), Lanes 9 and 11), confirming our above mapping result, that is, there were two overlapping BCEs within the epitope peptide of $hZP3^{301–320}$ (Figure 2(e) and Figure 5(b)). As shown in Figure 5, the rabbit serum to P1 or P2 diluted to 1 : 50 showed a strong positive reaction with the human ZP (I-J), and red fluorescence was absent when the oocytes were treated with preimmune serum (g-h). These results showed that production of specific immunoglobulin reactive with P1 or P2 epitope peptide and with human ZP was elicited by P1 or P2 peptide in immunized rabbits, respectively.

4. Discussion

The hZP3 protein has been an interesting target antigen for the development of a contraceptive peptide vaccine. Two $BCEs^{137–150 \text{ and } 334–341}$ on hZP3 were first identified with serum to each synthetic peptide based on computer prediction or sequence comparison of a known BCE sequence of mZP3 with other ZP3 protein in many mammals [15, 16]. The former 14 mer $peptide^{137–150}$ (P7) of hZP3, however, was not recognized by both sera to r-hZP3 in ELISA [17] and serum to r-hZP3a in Western blotting (Figure 1(b), Lane 5), suggesting that it might not be a self-epitope peptide of hZP3, although it could elicit antibody capable of binding to native hZP and its antiserum might be used as a marker for the identification of hZP3 protein [15]. For the latter 8 mer $peptide^{334–341}$ (P11) that elicited antibodies reacting to human ZP in transgenic mice [16], although it failed to be identified by serum to r-hZP3 in the study on epitope mapping of $hZP3^{22–360}$ [17], the result that peptide P11 could be recognized by serum to r-hZP3b in this study (Figure 1(b), lane 9) suggested that it should be an epitope of hZP3. As well, the 20 mer $peptide^{45–64}$ (P5) of hZP3 mapped by serum to r-hZP3 [17], it could not react to serum to r-hZP3b in this study (Figure 1(b), Lane 3). At present, we cannot explain these distinct mapping results, including the above-mentioned 8mer $peptide^{334–341}$, because both groups employed different antisera (against r-$hZP3^{22–460}$ and r-$hZP3a^{22–176}$, resp.).

The antigenicity and immunogenicity of $hZP3^{172–190}$ [17], $mstZP3^{301–320}$ [18, 26], and $bmZP3^{300–322}$ [27] were previously reported. These synthetic peptides elicited antibodies capable of reacting to native mstZP, bmZP, and hZP,

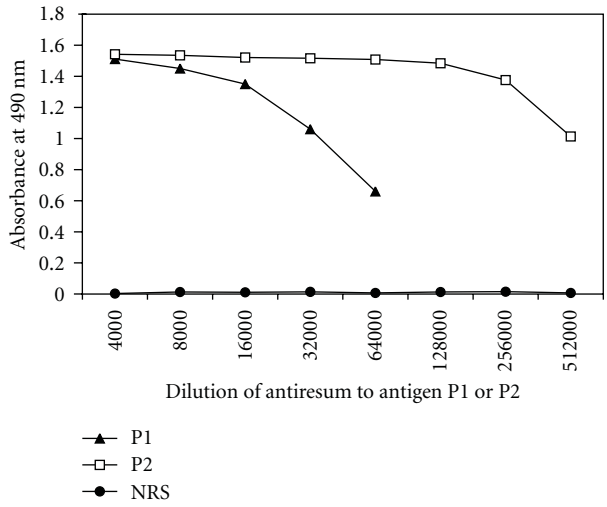

FIGURE 4: Comparison antibody titers to synthetic P1 and P2 of hZP3. The antibody levels were determined by ELISA using synthetic P1 (▲) and P2 (□) peptide as antigens. Normal rabbit serum (•) and or P3 (not shown) were used as negative controls. Titers were determined based on the highest dilution of the sample that generated OD greater 0.2.

respectively. The peptide sequence$^{301–320}$ is highly conserved between mstZP3 and bmZP3 sequences. The mst-serum tomstZP3$^{301–320}$ linked to a promiscuous helper TCE from tetanus toxoid showed up to 60% of inhibition in human sperm-zona binding in vitro [26]. Conversely, the bm-serum to bmZP3$^{300–322}$ conjugated to diphtheria toxoid failed to show any significant decrease in sperm-zona binding in hemizona assay [27]. The reasons for this discordance are not clear and more research is required to explain these findings. In the present study, because there is difference of one residue at the position^{305} of hZP3$^{301–320}$ (P), mstZP3$^{301–320}$ (A), and bmZP3$^{301–320}$ (S), its antigenicity and specificity was preliminarily evaluated using synthetic peptide P2 conjugated with KLH, and was compared with peptide P1. As shown in Figures 4 and 5, peptides P1 and P2 elicited high-titer antibodies reacting with native hZP and r-hZP3b peptide expressed in *E. coli*, but the antigenicity of the latter was significantly greater than that of the former. For the antigenic diversity of peptides P1 and P2, one possible explanation might be because there were antibodies against two overlapping BCEs (NSNWF$^{306–310}$ and EGP$^{313–315}$) within the peptide P2 sequence. As for the efficacy of each antiserum to inhibit human sperm-zona binding, it remains to be further evaluated by carrying out competitive hemizona or sperm-egg binding assays.

The serum to a synthetic peptide was often used to map minimal motif of identified BCE peptide [16, 28]. To further check the specificity of peptides P1 and P2 as antigen, the Western blotting was carried out with two sets of 8 mer peptides (P27∼P33 and P34∼P46). Surprisingly, the rabbit serum to P1 not only could recognize peptides P28 and P29, but also reacted with peptides P30 and P31 that were not recognized by rabbit serum to r-hZP3b, whereas the serum to P2 only recognized peptides P42 and P43, but did not recognize other peptides (P43–P46) reacted with serum to r-hZP3b (data not shown). The data suggested that their BCE motifs generated a "drifting" phenomenon when using serum against synthetic peptide P1 or P2 to map minimal motif compared with the result identified with serum to r-hZP3b, that is, the minimal motifs mapped with serum to a synthetic peptide and to a native or r-protein might be different sometimes.

The synthetic peptides method [29–31] has been often used to delineate linear BCEs on a protein; however, the number of mapped BCE always was less or some BCEs were missing when using serum to r-protein and ELISA. For instances the following hold. (1) Only 3 BCEs on hZP3, not including a BCE on its C-terminus transmembrane- like domain, were mapped with serum to CHO-expressed r-hZP3$^{22–460}$ [17] compared with the result of mapped 6 BCEs in this study. (2) It could not be defined how many BCEs there were in six of potential reactive neighboring 15mer-peptides (P5–P8, P11-P12, P14–P18, P31–P33, P38-P39, and P43-P44) in the epitope mapping of brushtail possum (bp) ZP2$^{40–634}$ with sera to r-bpZP2$^{40–311/305–634}$ [32], which might be one of causes why several BCE peptides could be missed or could not be defined in above two epitope mapping studies using synthetic peptide library. As we know, there may be only one BCE on mapped two neighboring overlapping peptides because they share a common sequence [33], but there may also be two or three BCEs according to our other epitope mapping results of E6, E7, and L1 proteins from HPV type 58 virus (data not shown). It obviously is a drawback to employ this method to map all BCEs on a protein, because it could not be used to carry out minimal motif identification of each reactive neighboring peptide with serum to r-protein, which is a way of answering how many BCEs there are on them.

Some studies on expression of single 4–12mer BCE peptides fused with Stv118 core protein [34] and epitope mapping with several purified short peptides fused with GST226 [35, 36], suggested the possibility of using biosynthetic peptides to map linear BCEs and their minimal motifs on a protein. However, not like the above studies using mAb, chicken sera to SARS-CoV, and SARS convalescent sera, it needs to solve a key problem to employ serum against r-protein, that is, how to avoid interfering of antibodies against some strong antigens from *E. coli* on distinguishing target blotted bands. Because any r-protein used as immunogen always contains a little bacteria proteins that could not be completely removed when purified at laboratory level. At present, this problem has been solved through using a truncated GST188 or Stv108 as carrier of short peptide expression. As showed in Figure 1, the blotted bands of the GST188-short peptides were to be located in a weak antigenic area of bacterial proteins on blotted membrane, which avoided two blotted bands of bacterial protein with 21 kDa and 31 kDa bands. Obviously, besides simple, cheap, reliable, and adaptable merits mentioned in our previous work [20], the present study showed another two distinct advantages of our improved biosynthetic peptide strategy: (1) it permits using serum against bacteria-expressed protein to map BCEs and their minimal motifs on

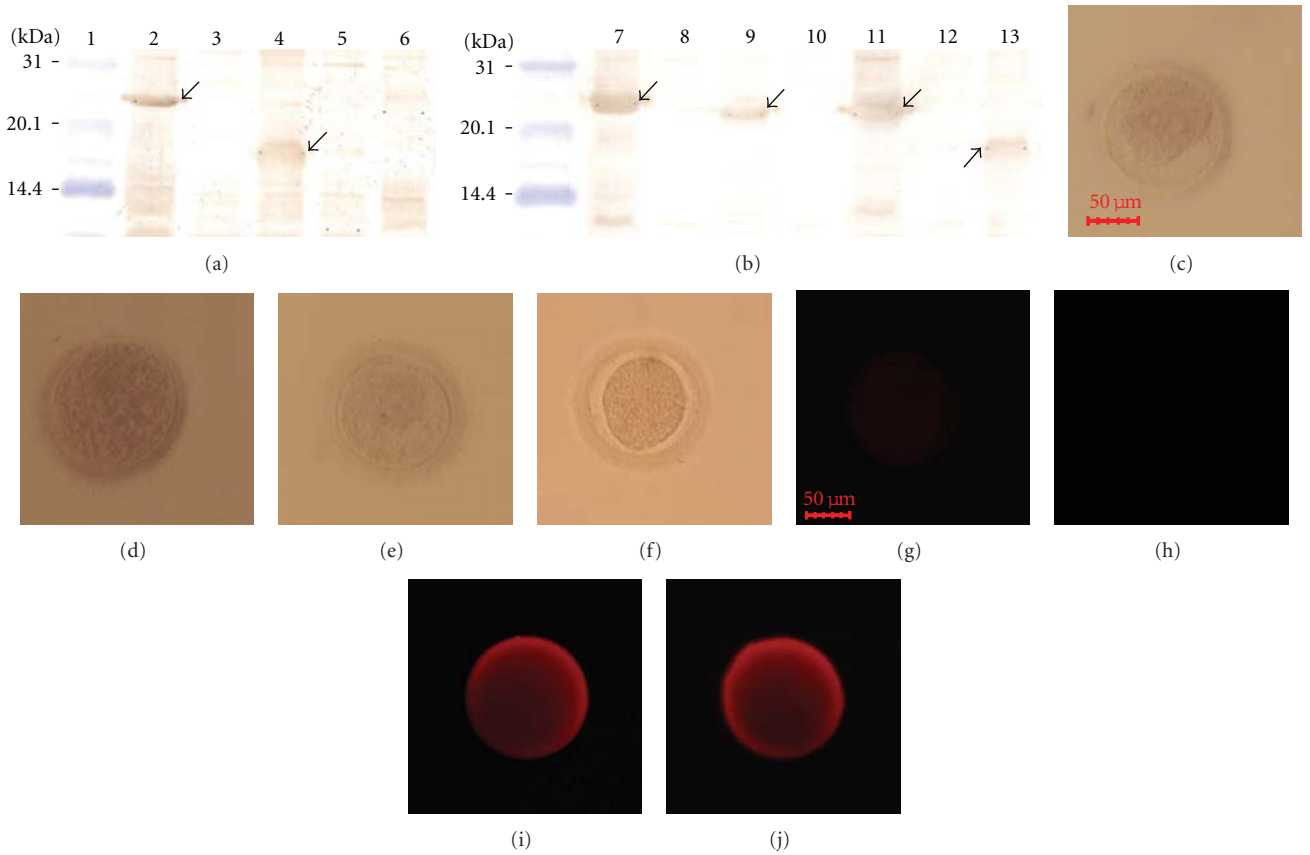

Figure 5: (a-b) Western blotting with sera to P1 or P2. Lane 1, prestained protein marker; Lanes 2 and 3, induced and uninduced GST-hZP3$^{172–190}$; Lanes 4 and 13, induced hZP3b$^{177–348}$; Lanes 5 and 6, induced Stv108 and GST188; Lanes 7 and 8, induced and uninduced GST-hZP3$^{301–320}$; Lanes 9 and 10, induced and un-induced GST-P37; Lanes 11 and 12, induced and un-induced GST-P44. (c–f) matching light images for the human oocytes imaged by immunofluorescence in panels g–j. (g–j) reactivity of rabbit sera to P1 or P2 with human oocyte by indirect immunofluorescence. Representative immunofluorescence patterns are shown for (g) preimmune serum; (h) immune serum only received CFA (i-j) immune serum to P1 or P2.

the target protein; (2) the 8mer–20mer peptide fusion proteins expressed by pXXGST-1 or pXXStv-3 plasmid could be used in Western blotting without purifying. In addition, the smallest binding motif of 3 residues for antibodies can be defined with biosynthetic peptides (Figure 2(e) and Figure 3), whereas the length mapped with synthetic peptides method was 4-5 residues [37].

In summary, this study is the first to utilize biosynthetic peptides and sera against r-hZP3a and/or r-hZP3b to map BCEs and their minimal motifs on hZP3 protein. The identification of five minimal motifs within four epitopes on hZP3 will help in developing contraceptive vaccines of multiepitope ZP peptides without ZP-specific TCE activities which may result in ovarian dysfunction for human use in future, and those defined BCE minimal motifs of hZP3 would be also used as specific probes to detect whether there are self-ZP antibodies to each BCE in the sera from patients with infertile and/or premature ovarian failure. Furthermore, the data presented here again clearly suggest that the biosynthetic peptide strategy employed in this study could be used to map all BCEs and precise linear epitopes on other entire ZP proteins in any species.

Acknowledgments

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Research Article

MBL Interferes with Endovascular Trophoblast Invasion in Pre-Eclampsia

Chiara Agostinis,[1] Fleur Bossi,[2] Elisa Masat,[2] Oriano Radillo,[1] Maddalena Tonon,[1] Francesco De Seta,[1] Francesco Tedesco,[2] and Roberta Bulla[2]

[1] *Institute for Maternal and Child Health, IRCCS "Burlo Garofolo", 34137 Trieste, Italy*
[2] *Department of Life Sciences, University of Trieste, Via Valerio 28, 34127 Trieste, Italy*

Correspondence should be addressed to Roberta Bulla, rbulla@units.it

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The spiral arteries undergo physiologic changes during pregnancy, and the failure of this process may lead to a spectrum of pregnancy disorders, including pre-eclampsia. Our recent data indicate that decidual endothelial cells (DECs), covering the inner side of the spiral arteries, acquire the ability to synthesize C1q, which acts as a link between endovascular trophoblast and DECs favouring the process of vascular remodelling. In this study, we have shown that sera obtained from pre-eclamptic patients strongly inhibit the interaction between extravillous trophoblast (EVT) and DECs, preventing endovascular invasion of trophoblast cells. We further demonstrated that mannose-binding lectin (MBL), one of the factor increased in pre-eclamptic patient sera, strongly inhibits the interaction of EVT with C1q interfering with the process of EVT adhesion to and migration through DECs. These data suggest that the increased level of MBL in pre-eclampsia may contribute to the failure of the endovascular invasion of trophoblast cells.

1. Introduction

The decidua is a newly formed tissue on the maternal side of human placenta and is characterized by active angiogenesis and structural modifications of the spiral arteries in the early phase of pregnancy. These changes, that include gradual loss of the musculoelastic structure of the arterial wall and replacement by amorphous fibrinoid material, are essential to create vessels of low resistance unresponsive to vasoconstrictive agents [1, 2] allowing continuous blood flow in the intervillous space.

An additional feature of the physiologic changes of spiral arteries is the endovascular invasion of extravillous trophoblast (EVT) that adheres to and replaces endothelial cells (ECs) giving rise to mosaic vessels in which trophoblast and ECs coexist [3]. Recently, we have provided data indicating that decidual endothelial cells (DECs) lining the spiral arteries acquire the ability to synthesize C1q. This protein binds avidly to the cell surface and acts as a physical link between endovascular trophoblast and DECs favouring the process of vascular remodelling [4]. C1q is a recognition molecules of the complement (C) system, one of the major components of humoral innate immunity, acting as a first line of defence against microbes. The C system can be activated via three pathways, namely, the classical, the alternative, and the lectin pathway, which are triggered by the three recognition molecules, C1q, C3, and mannose-binding lectin (MBL), respectively [5]. The system is also involved in the elimination of dead or modified self cells [6], but new roles in inflammatory, immunological processes, and tissue remodelling are emerging.

Failure of spiral artery to undergo transformation may lead to a spectrum of pregnancy disorders, including pre-eclampsia [7], foetal growth restriction, and miscarriage [8, 9]. Pre-eclampsia is a complication of pregnancy characterized by hypertension and proteinuria and develops in normotensive pregnant women after midgestation. Inflammation and innate immunity seem to play an important role in the aetiology of pre-eclampsia [10]. Several recent studies suggest an association between increased complement dysregulation and pre-eclampsia [11]. The role of the lectin pathway in the onset of this syndrome is a controversial issue.

The activity of MBL-MBL associated serin proteases (MASP)2 complexes is not increased in pre-eclamptic (PE) women [12]. A higher concentration of MBL has been demonstrated in the plasma of patients, compared to normal pregnant women [13] although the functions of this molecule in pregnancy remains to be clarified, despite the increased serum MBL concentration during pregnancy [14]. The association of a genetically related MBL polymorfism with MBL decreased functional activity has been reported to be protective against pre-eclampsia [15]. The level of MBL in the vaginal cavity changes during the menstrual cycle being produced locally by vaginal cells [16]. MBL seems to play an important role in embryo implantation since the analysis of uterine flushings, obtained at the time of oocyte retrieval for the in vitro fertilisation, revealed an increased level of MBL in patients with unexplained infertility compared with patients involved in IVF/ICSI for male or tubal infertility [17].

The aim of the present study was to evaluate the effect of sera obtained from pre-eclamptic patients on the process of vascular remodelling using in vitro models of trophoblast adhesion to and migration through DECs. We further investigate the ability of MBL to interfere with the process of trophoblast-endothelial cell interaction in order to define one possible mechanism responsible for the endovascular invasion failure in this severe multifactorial disease.

2. Material and Methods

2.1. Study Groups. In this study 11 pre-eclamptic and 11 normal pregnant women matched for gestation and parity were enrolled. The diagnosis of pre-eclampsia was established according to the standard criteria [18]. An informed consent was obtained from all women participating to the study. The study was approved by the Bioethical Committee of IRCCS, Burlo Garofolo, Trieste, Italy.

2.2. Collection and Processing of Sera and Measurement of MBL. Serum samples were obtained antepartum at the time of clinical diagnosis of the syndrome. The level of MBL in the sera was measured using the MBL oligomerELISA kit (Bioporto/Antibodyshop, Gentofte, Denmark).

2.3. Cell Isolation and Culture. EVT was purified from placental specimens after removal of decidual tissue and fetal membrane as previously described [3]. Briefly, placental tissue was incubated with HBSS containing 0.25% trypsin and 0.2 mg/mL DNase (Roche, Milan, Italy) for 20 min at 37°C. After fractionation through Percoll gradient, the leukocytes were totally removed by immunomagnetic beads coated with mAb to CD45 (Dynal, Invitrogen, Milan, Italy). EVT collected by negative selection were seeded in 25-cm^2 flask coated with 5 μg/cm^2 fibronectin (FN, Roche), cultured overnight in RPMI 1640 (Gibco, Invitrogen), supplemented with 10% FCS, and finally detached by trypsin-EDTA treatment. The cells obtained under these conditions contained 95% cytokeratin 7-positive EVT and a few vimentin-positive stromal cells. The presence of contaminating leucocytes and ECs was excluded by RT-PCR assay for CD45 and FACS analysis with anti-vWF and anti-CD31 antibodies, respectively.

DECs were isolated from decidual biopsy specimens as previously described with some modifications [3]. Briefly, the tissue was finely minced, digested first with 0.25% trypsin (Sigma-Aldrich, Milan, Italy) and 50 μg/mL DNase I (Roche) overnight at 4°C, and then with collagenase type 1 (3 mg/mL) (Worthington Biochemical Corporation, DBA, Milano, Italy) for 30 minutes at 37°C. The cells collected at the interface of Ficoll-Paque gradient (G&E Healthcare, Milan, Italy), after the centrifugation of the cell suspension at 690 ×g for 30 minutes, were positively selected with Dynabeads M-450 (Dynal, Invitrogen) coated with Ulex europaeus 1 lectin (Sigma-Aldrich). Cytofluorimetric analysis showed that more than 95% of the cells stained for vWF (Dako-Cytomation, Milan, Italy). The cells were seeded in 12.5-cm^2 flask precoated with 5 μg/cm^2 fibronectin (Roche) and maintained in endothelial serum-free basal medium (GIBCO, Invitrogen) supplemented with 20 ng/mL bFGF (basic fibroblast growth factor) and 10 ng/mL EGF (epidermal growth factor) (GIBCO, Invitrogen).

2.4. In Vitro Immunofluorescence Analysis. EVT or DECs were plated on 8-chamber culture slides (BD Biosciences Discovery Labware, Milan, Italy) coated with FN (10 μg/mL) at 37°C and left to adhere for 2 h. After fixation and permeabilization with FIX&PERM kit solutions (Invitrogen), EVT was stained with mAb OV-TL 12/30 anticytokeratin 7 (CK7, Dako-Cytomation, Milan, Italy), and DECs were stained with mAb BV9 antihuman VE-cadherin obtained through the courtesy of E. Dejana (Mario Negri Institute, Milan, Italy). The binding of these antibodies was revealed with goat antimouse FITC-conjugated secondary antibodies (Dako-Cytomation). Images were acquired using a Leica DM3000 microscope (Leica, Wetzlar, Germany) and the pictures were collected using a Leica DFC320 digital camera (Leica).

2.5. EVT Adhesion Assay. EVT adhesion assay was performed as previously described [19], with some modification. Briefly, 96-well plate was coated with 10 μg/mL of C1q (Quidel, Medical Systems, Genoa, Italy), MBL (kindly provided by Prof. Peter Garred, Department of Clinical Immunology, Rigshospitalet, University of Copenhagen, Copenhagen, Denmark), or BSA (Sigma-Aldrich) in bicarbonate buffer at 4°C overnight and blocked with 1% BSA for 1 hour at room temperature. Cells were fluorescently tagged with the lipophilic dye DiI (Molecular Probes, Invitrogen), resuspended in RPMI with 0.1% BSA, and then added to protein-precoated wells. Labelled EVT cells previously incubated with sera (1 : 50) or MBL (2 μg/mL) were added to C1q-coated wells for 45 min at 37°C in an air/CO_2 incubator. The number of adherent cells were counted with Infinite200 (ABS 544 nm, EM 590 nm) (TECAN Italia S.r.l.) with reference to a calibration curves established with increasing number of labeled EVT cells.

2.6. EVT/DEC Adhesion Assay. The assay to evaluate adhesion of EVT to DECs has been previously described [3].

To evaluate the effect of sera or MBL on the adherence of trophoblast to DECs, DECs grown to confluence on 96-microwell plates (Costar, Milan, Italy) were cocultured for 45 min at 37°C in an air/CO_2 incubator with an EVT cell suspension (10^5 cells/100 μL) labelled with a fluorescent dye (Fast DiI, Molecular Probes, Invitrogen) previously incubated with sera (1 : 50) or MBL (2 μg/mL). The nonadherent EVT cells were removed by washing with Dulbecco-PBS containing Ca^{2+} and Mg^{2+} (0.7 mM). The number of adherent cells were counted with Infinite200 (ABS 544 nm, EM 590 nm) (TECAN Italia S.r.l., Milano, Italy) with reference to a calibration curves established with increasing number of labeled EVT cells.

2.7. Transendothelial Migration Assay. DECs (2×10^4) were seeded onto 20 μg/mL fibronectin-coated polycarbonate insert of a 24-well FloroBlock Transwell system (6.5 mm diameter, 8-μm pores; BD Falcon) and used 5 days after plating [3]. EVT cell suspension (10^5 cells/100 μL) labelled with Fast DiI was added to the upper compartment of the transwell in the presence of sera (1 : 50) or MBL (2 μg/mL). EVT cells were allowed to migrate for 24 h in human endothelial serum-free medium supplemented with basic FGF, recombinant EGF (Gibco Invitrogen). Cells present in the lower chamber or adherent to the lower surface of the transwell insert were counted with Infinite200 (ABS 544 nm, EM 590 nm) (TECAN Italia S.r.l.) with multiple reads of same well and the number of migrated cells were expressed as percentage with reference to a calibration curves established with increasing number of labelled EVTs plated in the lower chamber.

2.8. MBL-Binding Assay. To evaluate the binding of MBL to EVT, 10^5 freshly isolated EVT cells were seeded onto 20 μg/mL fibronectin-coated 96-well plate. The cells were incubated with 0.1 μg/mL purified recombinant MBL, kindly provided by Professor Peter Garred, for 2 h at room temperature. The binding of the protein to the cells was analyzed using a monoclonal mouse antihuman MBL antibody (clone HYB131-01, Bioporto/Antibodyshop) 5 μg/mL, followed by AP-conjugated secondary antibodies (Sigma-Aldrich) 1 : 10000. The enzymatic reaction was developed with PNPP (p-nitrophenyl phosphate) (Sigma-Aldrich; 1 mg/mL) as substrate and read kinetically at 405 nm using a Titertek Multiskan ELISA reader (Flow Labs, Milano, Italy).

2.9. Statistical Analysis. Results are expressed as mean ± SD or as *box plot* graphs, in which the line in the middle of the box represents the median where the lower and the upper edges of the box are the 1st and 3rd quartile, respectively. Statistical significance was determined using Student's t test to compare two groups of data. Values of $P = 0.05$ or less were considered to be statistically significant.

3. Results and Discussion

3.1. Sera Obtained from PE Patients Affect Trophoblast-Endothelial Cell Interaction. We have previously demonstrated that trophoblast cells are able to adhere and migrate through endothelial cells [20]. A representative pattern of EVT cells and DECs used in the present investigation is shown in Figure 1. The effect of sera collected from PE and normal pregnant women at the same gestational age is presented in Figure 2(a). The results clearly show a significantly lower adhesion of EVT to DECs in the presence of pathological sera. The data were confirmed in at least three experiments using different preparation of trophoblast and endothelial cells. These results extend our previous observations obtained with sera from patients suffering from recurrent spontaneous abortion (RSA) [20]. These data suggest that endovascular invasion of trophoblast cells may be controlled by serum factors in several diseases associated with pregnancy failure.

To further confirm the contribution of sera obtained from PE patients to the process of vascular remodelling, we next examined the ability of these sera to influence the migration of EVT through DECs. To this end, FastDiI-labelled EVT cells were allowed to migrate through to the monolayer of DECs grown to confluence on the insert of a transwell system in the presence of decidual conditioned medium as a source of chemotactic factors. Under these condition, the number of migrating EVT cells was approximately 70% (data not shown). The migration rate increased to approximately 95% following the addition of sera obtained from normal pregnant women. Conversely, the sera from PE patients elicited a strong inhibitory effect and cell migration dropped to 35% (Figure 2(b)).

Since C1q synthesized by DECs during pregnancy acts as a physical link between endovascular trophoblast and DECs favouring the process of vascular remodelling [4], we decided to investigate the effect of serum from PE patients and control pregnant women. The results presented in Figure 2(c) show that the sera obtained from PE patients significantly reduce the adhesion of EVT cells to C1q.

3.2. MBL Present in PE Sera Is Responsible for the Failure of the Interaction of EVT to DECs. MBL is one of the complement components that undergoes changes in pre-eclampsia. Than et al. [13] have published data indicating that patients had higher levels of plasma MBL compared to normal pregnant women. We, therefore, wondered whether MBL may be one of the factors responsible for the serum effect on EVT cell interaction with endothelial cells.

We initially evaluated the MBL concentration of sera from PE patients and normal pregnant women used in the inhibition experiments. The data shown in Figure 3 clearly indicate that PE patients had a significantly higher level of serum MBL than control women, confirming the observation by Than et al. [13]. The increase in MBL in normal pregnancy with respect to the level found in nonpregnant women [14] and the further increase in MBL concentration in patients with pre-eclampsia, which is considered an inflammatory condition [21], is in line with the fact that MBL acts as an acute phase protein [22].

To investigate the effect of MBL in the interaction between EVT and DECs, EVT were incubated with 2 μg/mL recombinant MBL protein for 15 min at 37°C prior to be

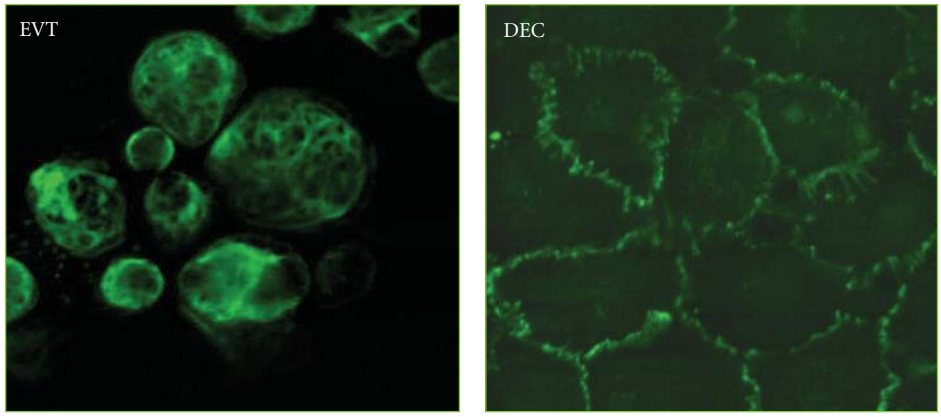

Figure 1: Immunofluorescence analysis of cells isolated from first trimester placenta. Purified EVTs were stained with mAb antihuman cytokeratin 7 (a) and DECs (b) with monoclonal anti-VE-cadherin. Images were acquired with Leica DM3000 microscope. Original magnification 100x.

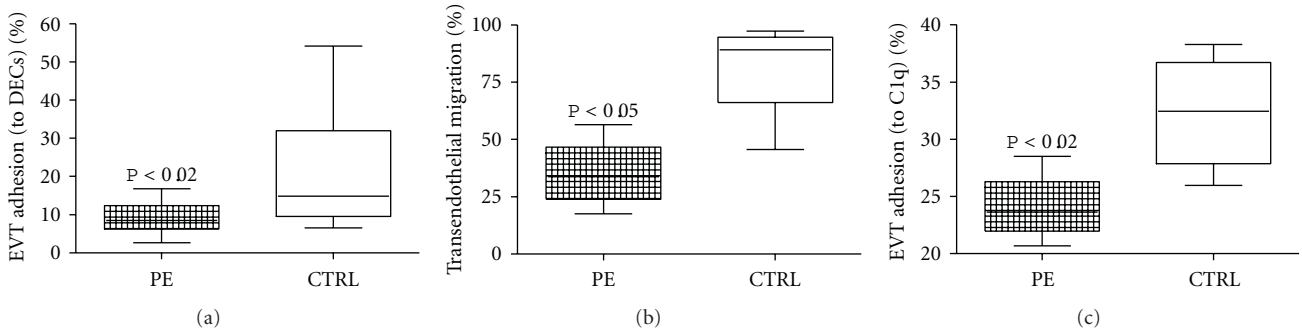

Figure 2: Effect of serum from pre-eclamptic and normal pregnant women in EVT/DEC interaction. Analysis of the effect of sera obtained from PE or normal women on the adhesion of EVTs to DECs (a), to C1q (c) and on transendothelial migration through DECs (b). EVTs were preincubated with PE or control sera (1 : 50). The results are expressed as percent of adhesion in reference to a standard curve. For each group, the line in the middle of the box represents the median. The lower and the upper edges of the box are the 1st and 3rd quartile, respectively.

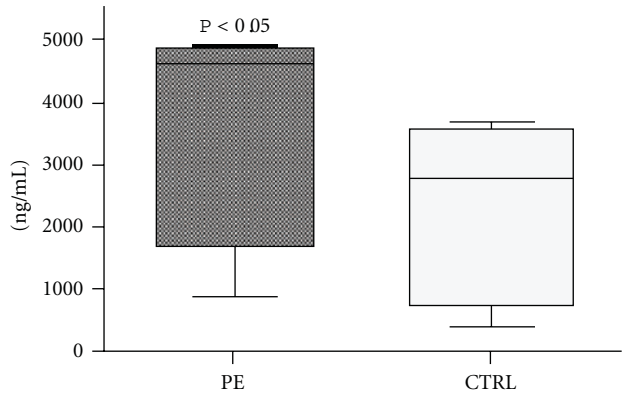

Figure 3: Analysis of serum levels of MBL in pre-eclamptic and control women. The level of oligomerized MBL in sera was measured using an MBL oligomer ELISA kit. The results are expressed as *box plot* graphs, in which the line in the middle of the box represents the median; the lower and the upper edges of the box are the 1st and 3rd quartile, respectively.

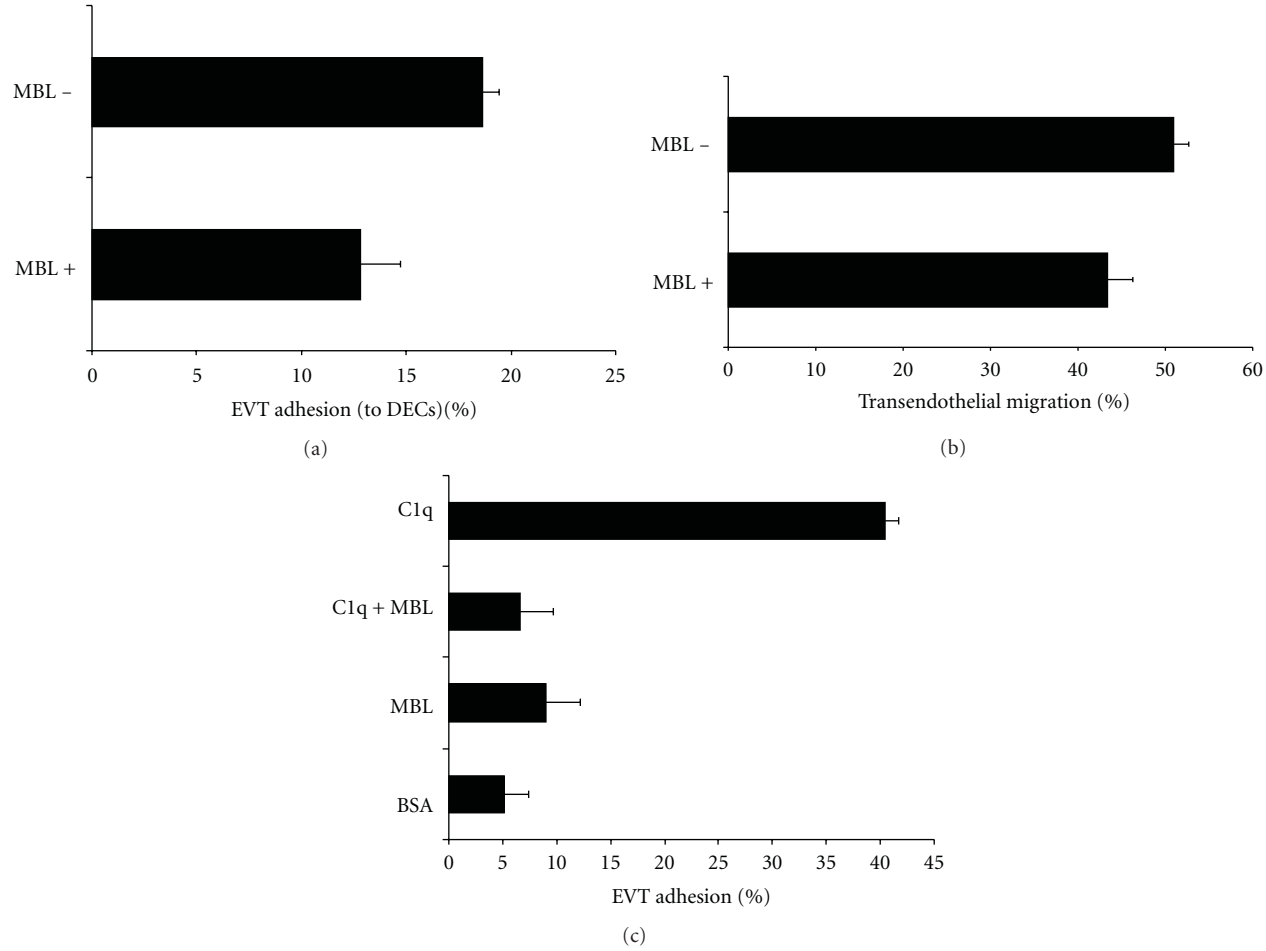

Figure 4: Analysis of MBL effect in the interaction between EVTs to DECs. To investigate the effect of MBL in the interaction between EVTs and DECs we performed adhesion (a) and migration assays through DECs, (b) in the presence of 2 μg/mL of MBL. The graph in (c) confirm the ability of EVT to adhere to C1q, while MBL was not able to promote adhesion of EVT as compared to BSA used as negative control. The ability of MBL to inhibit the adhesion process was also evaluated on the binding of EVT to C1q (C1q + MBL). $^{**}P < 0.01$; $^{***}P < 0.001$; $^{\S}P < 0.00001$.

tested in the adhesion and migration assays performed as described above. As shown in Figure 4, both adhesion to and migration of EVT through DECs were significant lower in the presence of MBL. The role of MBL was evaluated also in EVT-C1q interaction. The data represented in Figure 4(c) confirmed the ability of EVT to adhere to C1q. MBL failed to promote adhesion of EVT, compared to BSA used as negative control but was able to substantially inhibit the adhesion of EVT to C1q.

The binding site for MBL on EVT is still unknown. We have collected evidence indicating that MBL binds strongly to EVT (data not shown) and probably covers the binding site for C1q. The gC1q/p33 receptor for the globular head of C1q previously shown to be expressed on the surface of EVT [4] is unlikely to be involved in MBL-mediated inhibition of EVT-C1q interaction, since it is not an MBL receptor [23]. The only putative receptors for MBL are calreticulin and complement receptor-1 that bind to the tail domains of C1q and function also as receptors for MBL [23, 24]. These receptors, however, are present on ECs and interact with both MBL and C1q which compete with each other for their binding to the surface of ECs as shown by Oroszlàn et al. in cross-inhibition experiments [25]. Since DECs already express C1q on their surface, we postulate that MBL is probably unable to interact with EC membrane, though these data need to be confirmed.

4. Conclusion

In this study, we have shown that sera obtained from PE patients strongly inhibit the interaction between EVT and DECs, thereby controlling endovascular invasion of trophoblast cells, a fundamental process for the progression of pregnancy. MBL, one of the complement components shown to be present at increased levels in PE patient sera, is responsible for the inhibition of EVT adhesion to and migration through DECs. Our data also show that MBL interferes with the interaction of EVT with C1q expressed on DECs acting as a molecular bridge between endovascular trophoblast and DECs. The inhibitory effect of MBL on EVT adhesion to DECs is apparently unrelated to the ability of this complement component to activate the lectin pathway based on the

observation that the levels of MBL-MASP2 complexes, which represent activation products of this pathway, are essentially similar in PE and control groups, indicating that the lectin pathway activation plays only a minor role in complement activation during pre-eclampsia [12]. On the basis of our results, we suggest that MBL, which is present at increased level in PE patients, may be one of the factors interfering with the endovascular invasion of trophoblast cells. Overall these data further indicate that proteins belonging to the C system may play alternative roles promoting physiological processes and inducing tissue damage.

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Clinical Study

Association between Functional Polymorphisms of Foxp3 Gene and the Occurrence of Unexplained Recurrent Spontaneous Abortion in a Chinese Han Population

Zaigui Wu,[1] Zeshan You,[1] Cai Zhang,[1] Zhuyu Li,[1] Xiumei Su,[2] Xiuming Zhang,[3] and Yinguang Li[1]

[1] *Department of Obstetrics and Gynecology, the First Affiliated Hospital of Medical College, Sun Yat-Sen University, Guangzhou 510080, Guangdong, China*
[2] *Department of Obstetrics and Gynecology, People's Hospital of Chaozhou city, Chaozhou 515600, Guangdong, China*
[3] *Center of Stem Cell Biology and Tissue Engineering, Sun Yat-sen University, Guangzhou 510080, Guangdong, China*

Correspondence should be addressed to Zeshan You, youzeshan888@21cn.com

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Unexplained recurrent spontaneous abortion (URSA) is an alloimmune disease associated with the failure of fetal-maternal immunologic tolerance in which the regulatory T lymphocytes (Treg) play a pivotal role. It is well known that Forkhead box P3 (Foxp3) is a crucial regulatory factor for the development and function of Treg cells. It has also been established that deficiency of the Foxp3 gene suppresses the regulatory function of Treg cells. To determine if functional polymorphisms at the Foxp3 loci are associated with URSA in humans, we genotyped four common polymorphisms of Foxp3 gene in 146 unrelated URSA patients and 112 healthy women. The results showed that rs3761548A/C and rs2232365A/G polymorphisms were significantly associated with URSA. Additionally, we found that the allelic distribution of rs5902434 del/ATT in URSA group was slightly different from that in the control group. We conclude that functional polymorphisms of the Foxp3 gene may confer an important susceptibility to URSA in the Chinese Han population, probably by altering Foxp3 function and/or its expression.

1. Introduction

Recurrent spontaneous abortion (RSA), which is defined as two or more consecutive pregnancy losses before the 20th week of gestation from the last menstrual period, occurs in approximately 1% to 5% of women at reproductive age [1, 2]. Although many known causes of RSA including anatomic (15%), infectious (1%–2%), hormonal (20%), immunological (20%), and genetic (2%–5%) have been identified, a significant number of cases (approximately 40%–50%) do not have known causes, and these cases are called unexplained recurrent spontaneous abortion (URSA) [3]. It has been proposed that URSA belongs to an alloimmune disease associated with the failure of fetal-maternal immunologic tolerance [4, 5]. Treg cells play a critical role in the induction of a privileged tolerant microenvironment at the fetal-maternal interface [6].

Growing evidence [7–9] suggests that women with URSA had remarkably reduced frequencies of $CD4^+CD25^+$Treg cells in peripheral blood as well as in deciduas. The reduction of Treg cell in URSA patients is closely related to the decreased expression of Foxp3 [9]. Moreover, a reduced suppressive capacity of Treg cells has also been implicated in URSA patients [10]. It is the reduced numbers and/or functional deficiency of $CD4^+CD25^+$Treg cells that cause the predisposition to miscarriage.

Foxp3 is a master regulator gene for the development and function of Treg cells [11]. Deficiency of the Foxp3 gene impairs the suppressive function of Treg cells [12]. It has been reported that there are associations between Foxp3 gene polymorphisms and autoimmune diseases, such as systemic lupus erythematosus (SLE) [13], autoimmune thyroid diseases (AITDs) [14], type I diabetes (TID) [15], and allergic rhinitis [16]. However, the association between

Table 1: Primers used in the genotyping by PCR-SSP or PCR-RFLP.

Marker Method	Allele Enzyme	Forward primer	Reverse primer	PCR product Tm/°C
rs2232365	A	5′-CCCAGCTCAAG	5′-GGGCTAGTGAG	442 bp
PCR-SSP		AGACCCCA-3′	GAGGCTATTGTAA C-3′	
	G	5′-CCAGCTCAAGA	5′-GCTATTGTAACA	427 bp
		GACCCCG-3′	GTCCTGGCAAGTG-3′	
rs5902434	deletion	5′-ACCTTTAAGTCTTCTGCC	5′-TGATTATCAGCG	356 bp
PCR-SSP		ATTTATTCTATTATTT-3′	CACACACTCAT-3′	
	ATT	5′-CCTTTAAGTCTTCTGCCA	5′-TGATTATCAGCG	358 bp
		TTTATTCTATTATTA-3′	CACACACTCAT-3′	
rs3761548	PstI	5′-GCCCTTGTCTA	5′-CAGCCTTCGCCA	487 bp
PCR-RFLP		CTCCACGCCTCT-3′	ATACAGAGCC-3′	63°C
rs2294021	HaeIII	5′-CACACACAATCCAT	5′-ATCTCCATGCCCTA	429 bp
PCR-RFLP		CCCAGTCACCC-3′	AGAAGGCCACC-3′	59°C

Foxp3 polymorphisms and URSA has not been defined so far. Thus, the purpose of this study was to determine if functional polymorphisms at the Foxp3 loci were associated with URSA in humans. We will focus on the following four loci of Foxp3 gene: rs2232365A/G, rs5902434del/ATT, rs3761548A/C, and rs2294021T/C.

2. Materials and Methods

2.1. Subjects. A total of 146 unrelated URSA patients (age: 22–40 years with a median age of 29.1 years) were selected from the Center of Stem Cell Biology and Tissue Engineering, Sun Yat-sen University (Guangzhou, China). All patients were treated with immunization using paternal lymphocytes during the period between January 2009 and October 2010. All patients had histories of at least two successive miscarriages with unexplained etiology before 12 weeks of gestation, and there was no successful pregnancy record (with the same partner) before this treatment. The median of the number of miscarriages was 3.1. The diagnosis of "unexplained" abortion was made by the following practical guidelines [8]: (1) uterus and cervical abnormalities were excluded by pelvic examination, ultrasound, and a diagnostic hysteroscopy; (2) chlamydia and ureaplasma were excluded by Cervical mucus culturing; (3) chromosome problems were excluded by Karyotypes of abortion couples and abortuses; (4) luteal function defect, hyperprolactinemia, and hyperandrogenemia were excluded by comprehensive hormonal examinations; (5) endocrine diseases, for example, diabetes, hyperthyroidism, and hypothyroidism, were excluded; (6) autoimmune factors associated with systemic lupus erythematosus (SLE) and the antiphospholipid syndrome (APS) such as antinuclear antibodies (ANA), lupus anticoagulant (LA), and anticardiolipin antibodies (ACL) were tested in three consecutive visits every other month; (7) all male partners had normal semen status. The control group containing 112 women (age: 23–44 years old) with at least one live birth was derived from volunteers undergoing routine annual gynecological examination in the same hospital between 2009 and 2010 and there had been no history of spontaneous abortion, preterm labor, or preeclampsia. The subjects in the control group were also examined on the endocrine and immune factors to exclude individuals with diabetes, AITDs, SLE and APS. These stringent criterions described above were used for selection of each subject to exclude borderline cases and enhance the reliability of the data. Genomic DNA was extracted from peripheral blood mononuclear cells using AxyPrep TM blood DNA extraction Mini Kit (AXYGEN, Pittsburgh, Pa, USA) according to the manufacturer's instructions. Written consents of the study were obtained from all patients and the control population after the detailed information sessions with each individual. The study protocol was approved by the Ethics Committee of Sun Yat-Sen University.

2.2. Genotyping of rs2232365A/G and rs5902434del/ATT Polymorphisms. Genotypes of rs2232365A/G and rs5902434/ATT were determined by using the polymerase chain reaction (PCR) with sequence-specific primers (PCR-SSP, Table 1). PCR was performed in a volume of 30 μL, containing 1 μL (20 ng) of genomic DNA, 2 μL (10 pmol) of each primer, 15 μL of Takara Ex Tap Mix and 10 μL of ddH2O. A "touch down" procedure was applied after an initial preheating step for 1 min at 98°C. The PCR parameter for amplification of rs2232365 locus was as follows (the annealing temperatures for rs5902434 was 62°C, 57°C, and 51°C, resp.).

The amplified PCR products were analyzed using 1.5% agarose gel electrophoresis, stained with ethidium bromide and photographed.

2.3. Genotyping of rs3761548A/C and rs2294021T/C Polymorphisms. Genotypes of rs3761548A/C and rs2294021T/C were determined using PCR-restriction fragment length polymorphism (PCR-RFLP, Table 1) method. PCR was performed in a volume of 30 μL, with 1 μL (20 ng) of genomic DNA, 2 μL (10 pmol) of each primer, 15 μL of Takara Ex Tap Mix and 10 μL of ddH2O, The parameters for PCR include

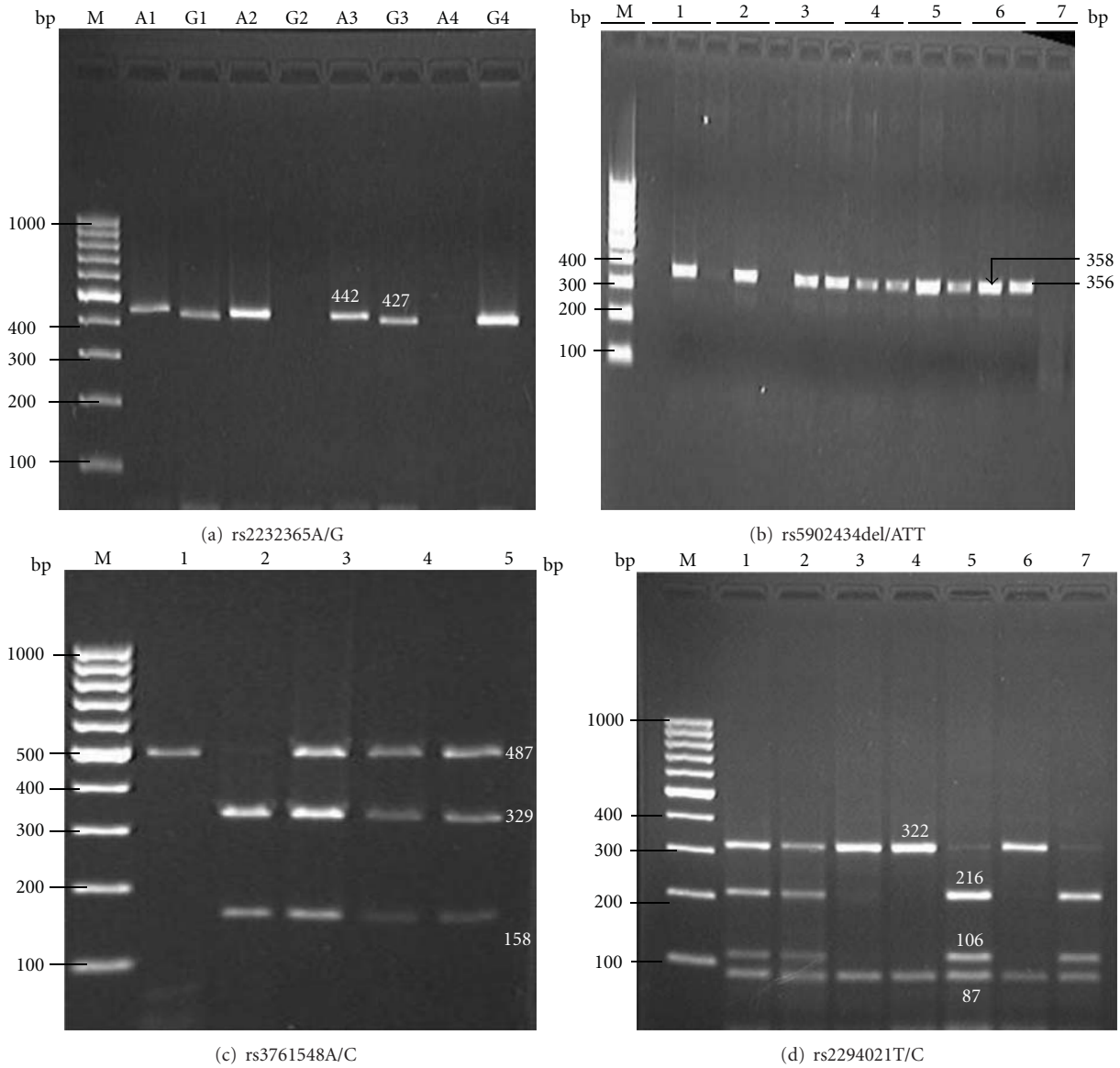

Figure 1: Typical band patterns of PCR-SSP or PCR-RFLP products. (a) is the band pattern for rs2232365. Lane 1 and 3 indicated A/G genotype; Lane 2 indicated A/A genotype; Lane 4 indicated G/G genotype. (b) is the band pattern for rs5902434. Lane 1, 2, and 3 indicated ATT/ATT genotype; Lane 4, 5, and 6 indicated del/ATT genotype; Lane 7 indicated del/del genotype. (c) is the band pattern for rs3761548. Lane 1 indicated A/A genotype; Lane 2 indicated C/C genotype; Lane 3, 4, and 5 indicated A/C genotype. (d) is the band pattern for rs2294021. Lane 1, 2 indicated T/C genotype; Lane 3, 4, and 6 indicated C/C genotype; Lane 5, 7 indicated T/T genotype. M represented 100 bp DNA marker.

an initial denaturing step at 98°C for 1 min, followed by 35 cycles of 98°C for 30 sec, annealing for 30 sec, and extension at 72°C for 1 min, and a final extension at 72°C for 7 min. A 15 μL aliquot was digested with 1μL restriction enzyme at 37°C for 16 hours and then separated on a 2% agarose gel. To ensure that the results were repeatable, a 10% sample of the subjects in the patient group and the control group were genotyped twice and the reproducibility was 100%.

2.4. Statistical Analysis. Hardy-Weinberg equilibrium (HWE) test, linkage disequilibrium test, and haplotype frequencies for pairs of alleles were estimated using software HAPLOVIEW (version 4.2). The genotype, allele frequency in URSA patients and control group were analyzed by standard Chi-square test. Unconditional univariate and binary logistic regression analyses were performed to obtain the odds ratio (OR) for risk of URSA at 95% confidence intervals (CI). All statistical analysis were performed using the SPSS 13.0 software package (SPSS, Chicago, I/l, USA).

3. Results

3.1. Identification of Genotypes of the Foxp3 Polymorphisms. For genotyping of rs2232365A/G and rs5902434del/ATT, each DNA sample was tested in duplicates with one reaction

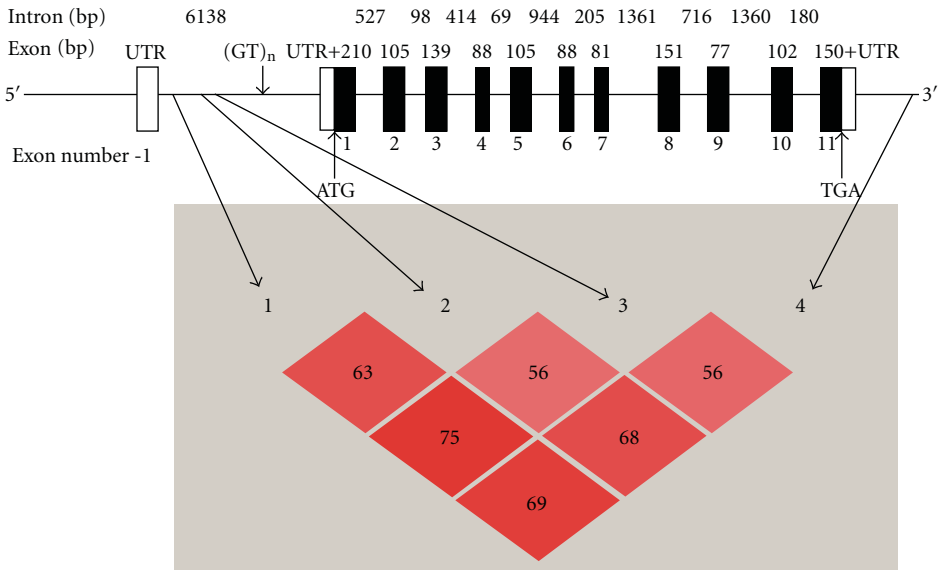

Figure 2: Schematic structure of the human Foxp3 gene and the linkage disequilibrium as determined in the URSA population (Haploview 4.20 version). The coding sequences, noncoding sequences and the introns were depicted as black boxes, white boxes, and horizontal lines, respectively. Exon numbers were shown below the exon boxes. The sizes of exons and introns were labeled above them. The locations of rs5902434 (1), rs3761548 (2), rs2232365 (3), rs2294021 (4), (GT)n microsatellite polymorphisms, the start cordon, and the stop cordon were indicated by arrows. The standard (D/LOD) was represented by red color and r^2 was presented as a number if it deviates from 100.

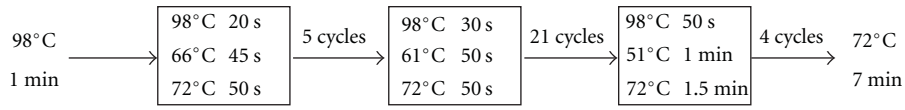

Figure 3

per allele of each locus. The A/A, A/G, and G/G genotypes had one (442 bp), two (442 bp and 427 bp), and one (427 bp) band(s), respectively. The del/del, del/ATT, and ATT/ATT genotypes also had one (358 bp), two (358 bp and 356 bp), one (356 bp) band(s), respectively. Genotypes of rs3761548A/C were defined by the presence of three different bands: A/A (487 bp), A/C (487 bp, 329 bp and 158 bp), and C/C (329 bp, 158 bp). For rs2294021T/C, genotypes were defined by the presence of five distinct patterns of bands: T/T (322 bp, 87 bp, and 20 bp), T/C (422 bp, 216 bp, 106 bp, 87 bp, and 20 bp), C/C (216 bp, 106 bp, 87 bp, and 20 bp). The 20 bp band was running out of the gel. The representative gels showing the typical patterns of bands were presented in Figure 1.

3.2. Genotypic and Allelic Distribution of the Foxp3 Polymorphisms in the Subjects of URSA and Control Groups. All the four SNPs were in HWE for both URSA group and control group with a P value from 0.08 to 0.85. In addition, these 4 SNPs were under linkage disequilibrium in the URSA group (Figure 2). The genotypic and allelic frequencies data were shown in Table 2. We performed statistical analysis to determine the association between each of the SNPs and URSA. There was no statistical difference between the genotypic and allelic frequencies of rs2294021T/C polymorphism in the URSA group and those in the control group. The genotypic frequencies of rs5902434 del/ATT polymorphism did not differ significantly between the URSA and control groups, but a weak association was observed in the allelic distribution ($P = 0.036$) between URSA and control groups. The genotypic and allelic distributions of rs2232365 A/G and rs3761548A/C were statistically different between the URSA and control groups. Consistent with the rs2232365 G allelic distribution, the frequency of the combined genotypes (A/G + G/G) in the URSA group (94.5%) was significantly higher than that in the control group (84.82%) ($P = 0.009$). The risk of URSA in women with G allele was higher than that in women with A allele (OR = 1.61, 95% CI = (1.11, 2.32)). Similar to the locus of rs2232365, the distribution of genotypes and alleles at the locus rs3761548 was also significantly different between the URSA and control groups.

3.3. Analysis of Haplotypes Based on the Three Polymorphisms in the Promoter Region. Because haplotypes are clusters of genetic variants and are inherited as a unit on the same chromosome, we stratified the subjects in URSA group and control group based on the Foxp3 promoter region haplotypes to explore the relative influence of each individual haplotype on URSA susceptibility. Of the 8 haplotypes (Table 3) defined by three polymorphisms at positions rs5902434, rs3761548, and rs2232365, they were classified into four distinct groups: ATT-C-A, ATT-A-A, del-A-G and del-C-G.

Table 2: Genotype and allele frequencies of the four Foxp3 polymorphisms in controls and URSA patients together with case-control analysis.

Marker	Genotype allele	URSA *N* (%)	Controls *N* (%)	χ^2 value	*P* value	OR (95% CI)
rs2294021	C/C	65 (44.50)	40 (35.70)			
	T/C	70 (47.90)	57 (50.90)			
	T/T	11 (7.60)	15 (13.40)	3.480	0.18[a]	
	C/C	65 (44.50)	40 (35.70)			
	T/C + T/T	81 (55.50)	72 (64.30)	2.037	0.154[b]	
	T/C	70 (47.90)	57 (50.09)			
	C/C + T/T	76 (52.10)	55 (49.10)	0.220	0.639[b]	
	T/T	11 (7.60)	15 (13.40)			
	C/C + T/C	135 (92.40)	97 (86.60)	2.401	0.121[b]	
rs5902434	ATT/ATT	11 (7.53)	14 (12.50)			
	del/ATT	62 (42.47)	56 (50.00)			
	del/del	73 (50.0)	42 (37.50)	4.620	0.10[a]	
	ATT/ATT	11 (7.53)	14 (12.50)			
	del/ATT + del/del	135 (92.47)	98 (87.50)	1.786	0.181[b]	
	del/ATT	62 (42.47)	56 (50.00)			
	del/del + ATT/ATT	84 (57.53)	56 (50.00)	1.450	0.229[b]	
	del/del	73 (50.0)	42 (37.50)			
	del/ATT + ATT/ATT	73 (50.00)	70 (62.50)	4.008	0.045[b]	0.60 (0.36, 0.99)
rs2232365	A/A	8 (5.48)	17 (15.18)	8.320	0.016[a]	1
	A/G	70 (47.94)	56 (50.00)	4.640	0.031[b]	2.66 (1.07, 6.61)
	G/G	68 (46.58)	39 (34.82)	8.260	0.004[b]	3.71 (1.47, 9.37)
	A/G	70 (47.94)	56 (50.00)			
	A/A + G/G	76 (52.06)	56 (50.00)	0.107	0.743[b]	
	G/G	68 (46.58)	39 (34.82)			
	A/A + A/G	78 (53.42)	73 (65.18)	3.607	0.058[b]	
	A/A	8 (5.48)	17 (15.18)			
	A/G + G/G	138 (94.52)	95 (84.82)	6.810	0.009[b]	3.09 (1.28, 7.44)
rs3761548	C/C	15 (10.27)	25 (22.32)	8.280	0.016[a]	1
	A/C	56 (38.36)	45 (40.18)	1.690	0.193[b]	1.44 (0.83, 2.47)
	A/A	75 (51.37)	42 (37.50)	8.620	0.003[b]	2.98 (1.42, 6.26)
	A/C	56 (38.36)	45 (40.18)			
	C/C + A/A	90 (61.64)	67 (59.82)	0.088	0.076[a]	
	A/A	75 (51.37)	42 (37.50)			
	C/C + A/C	71 (48.63)	70 (62.50)	4.920	0.027[b]	0.57 (0.34, 0.94)
	C/C	15 (10.27)	25 (22.32)			
	A/C + A/A	131 (89.73)	87 (77.68)	7.020	0.008[b]	2.51 (1.25, 5.03)
rs2294021	C	200 (68.50)	137 (61.20)			
	T	92 (31.50)	87 (38.80)	3.01	0.080[c]	1.38 (0.96, 1.99)
rs5902434	ATT	84 (28.77)	84 (37.50)			
	del	208 (71.23)	140 (62.50)	4.40	0.036[c]	1.49 (1.03, 2.15)
rs2232365	A	86 (29.45)	90 (40.18)			
	G	206 (70.55)	134 (59.82)	6.50	0.010[c]	1.61 (1.11, 2.32)
rs3761548	C	86 (29.45)	95 (42.40)			
	A	206 (70.55)	129 (57.60)	8.37	0.003[c]	1.73 (1.20, 2.50)

P^a was determined by Pearson's Chi-square test for 3×2 contingency tables.
P^b, P^c was determined by Pearson's Chi-square test for 2×2 contingency tables.
$P^a \leqq 0.05$, $P^b \leqq 0.017$, $P^c \leqq 0.05$ are considered statistically significant.

Table 3: Eight three-locus Foxp3 promoter region haplotypes identified in URSA group and the control group and their estimated frequencies using the software HAPLOVIEW (version 4.2).

Allele			Haplotype frequency (N, %)				
rs5902434	rs3761548	rs2232365	URSA	Control	χ^2 value	P	OR (95% CI)
del	C	A	6 (2.1)	8 (3.4)	0.824	0.36	
del	A	G	187 (64.1)	93 (41.6)	25.86	0.000	2.51 (1.75.3.58)
del	C	G	9 (3.2)	35 (15.8)	25.39	0.000	0.18 (0.08, 0.37)
del	A	A	5 (1.8)	4 (1.7)	0.013	0.91	
ATT	A	G	4 (1.3)	2 (0.7)	0.387	0.53	
ATT	A	A	10 (3.3)	30 (13.5)	18.6	0.000	0.22 (0.10, 0.46)
ATT	C	G	6 (1.9)	4 (1.6)	0.05	0.82	
ATT	C	A	65 (22.3)	48 (21.6)	0.033	0.86	

The frequencies of the latter 3 haplotypes (ATT-A-A, del-A-G, and del-C-G) were significantly different between URSA and control groups. The proportion of haplotype ATT-A-A and Del-C-G in URSA group was significantly lower than that in the control group, indicating that these haplotypes play a protective role in the occurrence of URSA, In contrast, haplotype del-A-G is a risk factor of URSA (OR = 2.51.95% CI = (1.75, 3.58)).

4. Discussion

Treg cells play a central role in the induction and maintenance of fetal-maternal immunologic tolerance. Development of Treg cells requires continued expression of Foxp3 [17], while attenuated Foxp3 expression results in its functional deficiency [18], We hypothesized that functional polymorphisms of the Foxp3 gene may contribute to the pathogenesis of URSA.

In the cases of the rs3761548A/C polymorphism, the A allele was a risk factor for SLE [13], AITDs [14], allergic rhinitis [16]. Interestingly, we found that genotypic frequency of rs3761548AA was significantly different between the URSA group and control group. Previous studies have shown that individuals with a genotype of rs3761548AA have the lowest production of Foxp3 among the three genotypes of this polymorphism [19]. Therefore, URSA patients with the AA genotype may have fewer Treg cells and/or weaker suppressive function and are difficult to achieve fetal tolerance, these results are consistent with previous reports, showing a decrease in the proportion of Foxp3+ Treg cells in URSA patients [8, 9]. Taken together, our results suggested that the rs3761548AA genotype may contribute to the occurrence of URSA.

For the polymorphism of rs2232365A/G, the distribution of alleles and genotypes differ significantly between the URSA group and control group. The risk of URSA in the women with G allele was higher than that in the women carrying an A allele (OR = 1.61, 95% CI = (1.11, 2.32)). These results are also consistent with the previous work [15], showing that Type I diabetes (TID) carrying a G allele has a higher occurrence of beta-cell failure. Because the functional differences between different genotypes are still unclear, we performed an extensive search for transcriptional factor-binding sites using TF-Search online tool (http://www.cbrc.jp/research/db/TFSEARCH.html). Interestingly, SNP variant of rs2232365A/G is located in a putative binding site for the transcription factor GATA-3, known to be essential for the Th2 immune response. More importantly, only when the A allele exists, can GATA-3 bind the promoter region of Foxp3. Th1/Th2 cytokine balance with Th2 predominance is a very important mechanism for the survival of the fetus in the maternal uterus [20]. It is also shown that Treg cells modulate the Th1/Th2 cell balance toward Th2 cell, and thus upregulate Th2 immune response [21]. Every recently, an intrinsic mechanism predisposing Foxp3-expressing regulatory T cells to Th2 conversion through GATA-3 in vivo has been identified [22]. Hence, we inferred that high frequencies of G allele and G/G genotype in URSA patients might decrease Th2 immune response and disrupt the balance of Th1/Th2, leading to a detrimental effect on the fetus during pregnancy.

The human Foxp3 gene is located in the small arm of the X-chromosome. Random inactivation of X-chromosome results in the presence of only two phenotypes for each polymorphism [23], because the phenotype of the heterozygous genotype is identical to that of a homozygous genotype. Therefore, we performed stratification analysis based on the possible phenotypes. Stratification of the URSA patients and controls by heterozygotes and homozygotes failed to detect any evidence that the heterozygotes were more frequent in URSA group, which was in contrast to the results from the previous studies [14, 16]. Among the 4 SNPs, however, the combinational genotypes del/del + del/ATT, A/G + G/G, C/C + A/C, and A/G + G/G appeared significantly different between the URSA and control groups, whereas only A/G + G/G and C/C + A/C were still significant after the Bonferroni correction for multiple testing of three tests($P \leqq 0.017$).These data, combined with the results from the haplotype analysis further provided evidence for the association between Foxp3 gene and URSA and supported that immune dysregulation is secondary to promoter heterogeneity in the Foxp3 gene. While some correlation has been found in the study, it is still very important to examine a larger number of samples from different populations and to investigate the proportions of Treg cells and Foxp3 expression or even

their suppressive capacity in peripheral or decidua among different genotypes in URSA patients to obtain more reliable conclusions.

5. Conclusions

We have evaluated the role of Foxp3 gene functional polymorphisms in the pathogenesis of URSA. Two functional polymorphisms in Foxp3 gene (rs2232365A/G and rs3761548A/C) are related to the occurrence of URSA. These results highlight the important role of Foxp3 in successful pregnancy.

Conflict of Interests

The authors have no financial conflict of interests.

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