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Immunology of Gametes and Embryo Implantation

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Preface

Infertility is an increasing problem in many societies. The success rate of assisted reproduction is still below 30%, although the treatments and techniques advance and improve continuously. One aspect of fertility and infertility may have been underestimated in the past: the immunology of gametes and the early embryo.

This book provides a wide overview of the current research in this field. Prominent and leading international groups contributed reviews of the most significant findings regarding this topic.

Sperm is a potential target for autoimmunity in men and alloimmunity in women, but physiologically, such immunoreactions do not occur. The preimplantation embryo is semiallogeneic to the mother, but again no destructive reaction occurs. Furthermore, the mother does not only develop a simple immunotolerance, but her uterus prepares a unique, exactly balanced immunological environment around the implantation site, which is indispensable for successful early pregnancy. The regulation of embryo implantation and trophoblast invasion through cytokines, growth factors and other pregnancy-related immunoregulatory molecules are described in detail. Autoimmunity and allergy are examples presented as causes of disequilibrium.

This volume presents the most recent knowledge about physiological and pathological immune reactions which favor or disturb fertility. It is an introduction for those readers who are not yet familiar with this field of immunology and updates the knowledge of specialists.

The general political and public interest in the field is reflected by the establishment and support of a European Network of Excellence entitled EMBIC (Embryo Implantation Control; www.embic.org; 2004–2008), which is strongly supported by the European Union. Several of the authors of this book are partners of the EMBIC.

Udo R. Markert

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Immune Privilege and Inflammation of the Testis

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Abstract

Immune cells are found in considerable numbers within the normal, unaffected testes of mammals, including humans. Located in the interstitial compartment, they are implicated in the mechanisms that make the testis an immunologically privileged site where germ cells are protected from autoimmune attack and foreign tissue grafts may survive for extended periods of time. With regard to normal development and function of the testis, both pro- and anti-inflammatory cytokines have been shown to play an important regulatory role. The testicular environment, however, does not preclude immune activation resulting in inflammatory reactions and potential damage. In experimental animals, active immunization with testicular tissue or adoptive transfer of specific T lymphocytes causes autoimmune orchitis. In men, infection and inflammation of the reproductive tract including the testes are widely accepted as important etiological factors of infertility. Whereas symptomatic orchitis due to bacterial or viral infections is considered to be rare, a high prevalence of asymptomatic testicular inflammatory reactions could be demonstrated among infertile males. Despite the patchy distribution of the lesions, inflammation is associated with disruption of testicular function, i.e. spermatogenesis. The pattern of lymphocyte infiltration and concomitant damage of seminiferous tubules supports the concept that activation of autoreactive T cells is involved.

Introduction

For many years, research on physiology and pathology of the testis has concentrated on the two major functions: the generation of male gametes and the production and controlled release of sex steroids. Interactions between the male reproductive tract and the immune system have been a source of both considerable curiosity and ignorance [1]. Studies in experimental animals indicate that the testis is one of very few organs of the body capable of sustaining

foreign grafts for extended periods of time without evidence of rejection [2, 3]. This 'immunological privilege' of the testis is believed to arise from the need to prevent immune responses against meiotic and haploid germ cells expressing 'nonself' antigens which first appear at the time of puberty, long after the establishment of self-tolerance in the perinatal period. Paradoxically, it is the same antigens that may become targets of a vigorous autoimmune attack if activation of specific T lymphocytes is induced elsewhere in the body [4, 5]. Furthermore, defense mechanisms including both innate and adaptive immunity are not generally impaired in the testis. This is illustrated by the obvious capacity of the testis for inflammatory responses to local and systemic infection [6, 7]. Although the complex mechanisms are not yet completely understood, there is considerable evidence that organ-specific immune regulation plays a key role in testicular function.

Immunobiology of the Testis

Immune Cells in the Testis

The testis, in spite of its immunologically privileged status, is not isolated from the immune system. Thus, immune cells are found in considerable numbers within the interstitial compartment of the normal unaffected testis of mammals, including humans [5, 8, 9] (table 1). In addition to resident macrophages, which represent the second most abundant cell type next to Leydig cells, mast cells are regular components of the interstitial and peritubular tissue [10, 11]. The number of lymphocytes in the testis is relatively small, although circulating immune cells have access to the organ and testicular lymphatic vessels allow drainage to regional lymph nodes [12, 13]. The presence of natural killer cells known to be involved in innate immune responses was reported in rodents, whereas consistent data for the human testis are not available. Moreover, dendritic cells as potential professional antigen-presenting cells and key players during induction of specific immune responses remain to be identified in the normal testis. Under physiological conditions, neither resident nor circulating immune cells are found within the seminiferous tubules and polymorphonuclear leukocytes remain completely absent.

There is substantial evidence that testicular macrophages and their functions are largely determined by the local environment [5, 14]. In the rat testis, two distinct subpopulations of macrophages could be identified by means of the monoclonal antibodies ED1 and ED2, with 85% of the cells revealing the 'resident' phenotype ED1–ED2+ [15]. The number of macrophages increases during pubertal development and is partly dependent on interaction with Leydig cells [14]. On the other hand, resident macrophages have a trophic effect on Leydig

Table 2. Putative mechanisms of testicular immunoregulation

Partial segregation of germ cell-specific antigens by the blood-testis barrier
Local anergy of T lymphocytes
Apoptosis of T lymphocytes (e.g. Fas/FasL-mediated)
Suppression of T cell-mediated immune responses by local mediators (e.g. cytokines)

intertubular connective tissue (CD4+ > CD8+) of the rete testis [8, 9]. As in rats, B cells are largely missing. With regard to lymphocyte functions in the noninflamed testis, especially subpopulations of T cells and their cytokine profiles remain to be characterized.

Possible Mechanisms of Testicular Immunoregulation

Prevention of germ cell-specific autoimmune reactions in the adult testis has long been explained solely on the basis that all germ cell-related autoantigens are segregated within the seminiferous tubules (see table 2). With the onset of meiosis during puberty, the so-called blood-testis barrier separates the basal compartment of the seminiferous epithelium containing spermatogonia and preleptotene spermatocytes from the adluminal compartment, where meiosis and spermiogenesis occur [for a review, see 20]. Morphologically, Sertoli cells are connected to each other over large areas of their surfaces by 'occluding tight junctions', which render intercellular spaces even impermeable to small molecules. Thus, the microenvironment of the adluminal compartment is isolated from the vascular system and circulating immune cells. However, segregation of germ cell-specific autoantigens by the blood-testis barrier is not complete. Autoantigenicity of the basal compartment of the seminiferous epithelium could be demonstrated in rats [21, 22]. Moreover, barrier functions are less extensive along the rete testis and excurrent ducts, where T cells are physiologically found within the lining epithelium [8].

Hence, tissue barriers and mechanical sequestration are important but not sufficient to protect male germ cells from autoimmune attack. There is considerable evidence that multiple immunoregulatory mechanisms are involved in maintaining both tolerance towards germ cells and immune privilege within the normal adult testis (table 2). While clonal deletion of autoreactive T lymphocytes through thymic selection during perinatal life does not control germ cell-related autoreactivity, mechanisms of peripheral tolerance such as local anergy of T cells have been considered to play a key role [5, 23]. Naïve T cells remain refractory to antigen-specific activation when encountering antigenic peptide:MHC complexes without antigen-independent costimulatory signals delivered by the same antigen-presenting cell [19]. In line with this concept,

constitutive expression of MHC molecules is found in the interstitial compartment of the testis, whereas costimulatory molecules such as ICAM-1, CD80, and CD86 are absent [4, 5, 24, 25].

Avoidance of deleterious autoimmune responses can also be achieved by active suppression mediated by regulatory T (T_{reg}) cell populations [19, 26]. Among CD4+ effector T cells, the cytokine profile produced by T_H2 cells exerts inhibitory effects on T_H1 cells which mediate cellular immune responses including organ-specific autoimmunity [19]. Preliminary observations in the normal murine testis suggest functional polarization of T cells towards a T_H2 profile [27]. Control of inflammation in vivo has also been attributed to T_{reg} producing IL-10 or transforming growth factor-β (TGF-β) [26]. Moreover, recent experiments with peripheral blood lymphocytes from healthy donors showed that the expansion of autoreactive T cells directed against a testis-related antigen can be suppressed by CD4+ CD25+ T_{reg} [28]. However, the presence and possible role of CD4+ CD25+ T_{reg} in the testis in vivo remains to be elucidated.

A further level of protection represents activation-induced apoptosis of T lymphocytes entering the immunologically privileged testis (table 2). Recent data obtained in a mouse model indicate that memory CD8+ T cells migrating into the testis are capable to mount an immune response against foreign tissue grafts but undergo apoptosis at an increased level via upregulation of Fas (CD95) and CD30 on their surface [29]. Indeed, expression of the ligand of Fas (FasL) by Sertoli cells has been implicated in maintaining testicular immune privilege as well as enhanced survival of allogeneic grafts cotransplanted with testicular tissue into other sites [23]. However, this hypothesis is stirring an ongoing debate and conflicting results including the human testis have been reported.

Finally, immunosuppressive activity has been described in testicular fluids [1, 5, 23]. There is evidence that locally produced mediators, i.e. cytokines, could play a key role in preventing immune activation and subsequent inflammation in the testis [1] (table 2).

The Dual Role of Cytokines in the Testis

Apart from overall hormonal control, precise regulation of spermatogenesis and steroidogenesis within the testis depends on numerous autocrine and paracrine mediators including growth factors and cytokines [30]. Under physiological conditions, resident macrophages as well as nonimmune testicular cells have been shown to produce both pro- and anti-inflammatory cytokines such as IL-1, IL-6, TNF-α as well as members of the TGF-β family [1]. The apparent overlap between testicular and immune regulatory functions of these cytokines could provide the key to understanding the phenomenon of immune

privilege and the processes leading to inflammation-mediated damage in the testis (see table 2).

The archetypical proinflammatory cytokine IL-1 (occurring as two isoforms: IL-1α, IL-1β) is abundantly secreted by activated macrophages, but is also inducible in other cell types [19]. In the rat testis, IL-1α is produced and secreted under physiological conditions by Sertoli cells [31, 32]. There is some evidence that spermatocytes and spermatids may also produce IL-1α constitutively [33]. High-affinity IL-1 binding sites and mRNA for the IL-1 signaling receptor have been found in most cells of the interstitium and seminiferous epithelium [34]. Testicular IL-1 is thought to play an important role in coordinating Sertoli and germ cell development within the seminiferous epithelium, and in controlling steroidogenesis [1, 35]. Recent data suggest that IL-1 generally inhibits LH-stimulated testosterone production, but can stimulate basal steroidogenesis under appropriate conditions [36]. In contrast to IL-1α, IL-1β does not appear to be produced in significant amounts in the normal testis [1]. However, as a third member of the family, IL-1 receptor antagonist has been shown to be produced by mouse Sertoli cells [37].

In the normal murine testis, expression of the cytotoxic immune effector molecule TNF-α has been found in pachytene spermatocytes and round spermatids [38]. Moreover, TNF-α is produced by activated testicular macrophages in vitro [39]. Similar to IL-1, TNF-α inhibits Leydig cell steroidogenesis [34], and its localization to the postmeiotic germ cells also indicates possible involvement in the process of spermatogenesis. Observations from the human testis suggest that TNF-α might play a role in controlling the efficiency of spermatogenesis, inhibiting germ cell apoptosis by regulating the level of FasL [40].

The macrophage migration inhibitory factor (MIF) is a pleiotropic protein with a wide tissue distribution participating in inflammatory responses and acting as a counterregulator of glucocorticoid-induced immune suppression [1]. In the rat testis, MIF has been localized to the Leydig cells [41]. Whereas testicular macrophages remain MIF negative, previously negative Sertoli cells revealed a significant compensatory MIF expression after ethane dimethane sulfonate treatment for Leydig cell ablation [42]. Furthermore, MIF has been found to reduce inhibin secretion by Sertoli cells in culture and to evoke a transient increase in calcium levels in peritubular cells [41, 43]. These data support a role for MIF in the paracrine regulation of Leydig cell-seminiferous tubule interactions. MIF has been shown to downregulate TGF-β2 secretion in peritubular cells. Hence, upregulation of the proinflammatory cytokine MIF during inflammation can inhibit the immunosuppressive response of the testis [44].

The TGF-β family members are dimeric cytokines with predominantly immunosuppressive and anti-inflammatory activities [19]. There are three

mammalian TGF-β isoforms (1–3), which are very highly expressed by Sertoli cells, peritubular cells and Leydig cells in the fetal and immature testis, although production declines dramatically postpuberty [45]. The receptors for TGF-β are found in both somatic and germ cells [46, 47]. Consequently, these cytokines have been implicated in controlling both Leydig cell and seminiferous tubule development [1]. A precise role in the adult testis has yet to be established, although TGF-β has been proposed as important factor of immune privilege [1, 23].

Inflammation of the Testis

Experimental Orchitis

The testicular environment does not preclude immune activation and potential damage. In fact, germ cells and their 'nonself' antigens elicit a vigorous organ-specific immune response when encountered outside the genital tract, e.g. after subcutaneous injection [for a review, see 4]. Experimental autoimmune orchitis (EAO) with characteristic deterioration of spermatogenesis ('aspermatogenesis') was first demonstrated in guinea pigs immunized with homologous testis homogenates in complete Freund's adjuvant [48]. Early perivascular infiltrates comprising lymphocytes and macrophages were followed by extensive peri- and intratubular infiltration of inflammatory cells. EAO is also inducible in other species and has been reported after injection of viable syngeneic germ cells without adjuvant in mice [49, 50]. Moreover, inflammation of the contralateral testis was observed after unilateral induction of experimental bacterial orchitis [51]. In various EAO models, testis-specific delayed-type hypersensitivity as well as autoantibody formation could be demonstrated [4]. Susceptibility to EAO appeared to be genetically determined by both MHC and non-MHC genes [4].

Concerning testicular immunopathology, T lymphocytes predominate inflammatory infiltrates in EAO, followed by B cells and nonresident macrophages [50, 52]. Notably, TNF-α-producing CD4+ Th1 cells have been implicated as key players in the development of EAO [53, 54]. In line with these results, EAO can be adoptively transferred into syngeneic recipients by CD4+ T cells or testis-specific T cell lines, whereas depletion of CD4+ T cells in vivo inhibits the disease [4]. Depletion of γ:δ T cells significantly increased inflammatory disease in the murine testis [51]. With regard to recruitment of leukocytes, chemokines and their receptors have to be considered as crucial components [55]. In contrast, the pathophysiological significance of early immune complex deposition in the basal compartment of seminiferous tubules remains to be elucidated.

Inflammation of the Human Testis

In men, infection and inflammation of the reproductive tract including the testes are widely accepted as important etiological factors of infertility [56]. However, most studies focussed on the excurrent ducts and male accessory glands, whereas the immunopathology of the testis received only little attention. Orchitis is considered to be a rare disorder, although precise epidemiological data are not available [6, 7]. The clinical entity 'orchitis' is particularly attributed to acute, symptomatic disease due to local or systemic infection, whereas subacute or chronic, asymptomatic inflammation of the testis including noninfectious disease is difficult to diagnose and therefore likely to be neglected.

Infectious orchitis is classified according to etiological and morphological aspects [6, 7]. The retrograde ascent of urethral pathogens, i.e. bacteria such as *Chlamydia trachomatis* or *Escherichia coli*, produces epididymoorchitis secondary to epididymitis, whereas orchitis associated with systemic, most commonly viral infections has been attributed to blood-borne dissemination of the pathogens. The classical example of this type of orchitis evolves as a complication of peri- or postpubertal mumps [7]. The so-called granulomatous orchitis describes specific testicular involvement in patients with tuberculosis, lepra, brucellosis, or related infections [6, 7]. Histolopathology of acute bacterial epididymoorchitis reveals edema and massive infiltration of both interstitial compartment and seminiferous tubules with mainly neutrophils [6]. Viral orchitis is characterized by multifocal perivascular as well as peri- and intratubular infiltrates with neutrophils, lymphocytes, plasma cells, and macrophages [6]. Affected seminiferous tubules show degeneration of the germinal epithelium sparing few spermatogonia and the Sertoli cells; concomitant thickening of the lamina propria may end in complete hyalinization and fibrosis of the tubules. Interestingly, chronic inflammatory reactions following acute orchitis are characterized by multifocal lymphocytic infiltrates and the aforementioned tubular changes, which are typical morphological features of EAO [6, 27]. Comparable to EAO in animals, severe inflammation of the human testis results in complete disruption of spermatogenesis as reflected by testicular atrophy and persistent infertility [7, 27]. Leydig cells in the interstitial compartment show little evidence of attrition in most patients; however, bilateral orchitis may lead to a failure of testicular androgen production [7].

With regard to noninfectious inflammation of the testis, testicular seminoma is almost invariably associated with extensive inflammatory infiltrates suggesting immune activation induced by the neoplastic process [57, 58]. Infiltrates contain mainly T lymphocytes with predominance of CD8+ T cells inside the tumor, whereas macrophages are found in the fibrovascular septa and at the periphery. Lymphocytic infiltrates are also observed around seminiferous tubules containing carcinoma in situ cells or in the contralateral testis

accompanying unilateral neoplasia [58, 59]. Moreover, manifestation of systemic vasculitis involving blood vessels of both testis and excurrent ducts has to be considered [6]. Granulomatous orchitis may occur as a primarily chronic, painless disease of unknown etiology or rare manifestation of sarcoidosis [7]. In contrast to respective animal models, 'traumatic orchitis' is not well characterized as a clinical entity in men. However, chronic inflammatory reactions were observed after herniotomy in both ipsi- and contralateral testis and have been classified as autoimmune orchitis [60].

So far, only few studies investigated testicular inflammatory reactions among infertile males. The reported prevalence of inflammatory, mainly lymphocytic infiltrates in testicular biopsies was 4.8–16.6% [59, 61, 62]. In a recent systematic reexamination of tissue specimens obtained from 260 asymptomatic patients with impaired fertility, testicular infiltration of lymphocytes was recorded even in 56.3% of the cases [27]. Histopathological changes ranged from single lymphocytes widely scattered in the interstitial compartment to dense peritubular and perivascular infiltrates distributed in a focal or multifocal pattern. Notably, the majority of infiltrates showed a peritubular localization. In addition, the degree of lymphocytic infiltration correlated with characteristic signs of tubular damage including partial or complete loss of germinal epithelium, thickening of the lamina propria, and complete tubular fibrosis. In line with data from the above-mentioned animal models, infiltrating activated lymphocytes are predominantly CD4+ and CD8+ T cells, which are accompanied by increased numbers of nonresident CD68+ macrophages and mast cells [8, 27]. For the latter two cell types, a shift from the interstitium to the seminiferous tubules was also reported for other testicular pathologies and has been associated with tissue remodeling and fibrosis [27, 63, 64].

Despite the patchy distribution of the lesions, testicular inflammatory reactions in infertile men are associated with significantly reduced testicular volume and score counts for spermatogenesis, when inflammation represents the primary disorder [27]. Serum FSH levels are not markedly increased in these cases compared to patients with testicular obstruction and preserved spermatogenesis. Interestingly, the prevalence of peritubular infiltrates in other testicular disorders closely correlates with the degree of tubular damage, i.e. impairment of spermatogenesis.

The hallmark of chronic inflammatory reactions in the human testis following acute bacterial or viral orchitis as well as those observed among infertile men, i.e. infiltration of activated inflammatory T lymphocytes, indicates a profound disturbance of the local immunoregulation and, thus, testicular immune privilege. Infiltrating inflammatory cells obviously overcome the immunosuppressive influence in the testis [5]. Both predominant peritubular localization of lymphocytes and characteristic morphological changes of the seminiferous

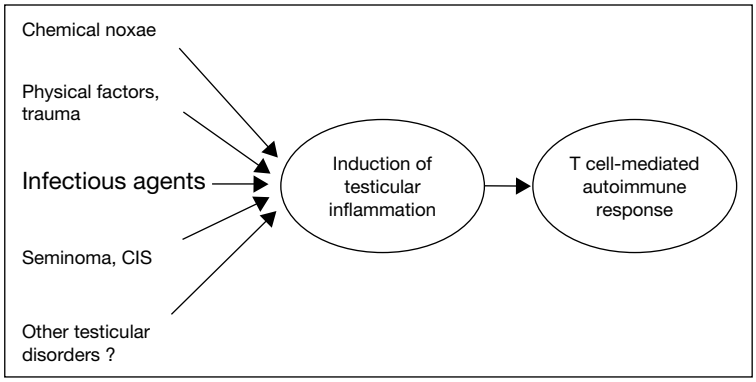

Fig. 1. Etiological factors and pathogenesis of chronic, asymptomatic testicular inflammatory reactions in infertile males – a hypothesis. CIS = Carcinoma in situ.

tubules resembling EAO support the concept that concomitant activation of autoreactive T cells is involved (fig. 1). It is unlikely, however, that deterioration of spermatogenesis results from direct T cell-mediated cytotoxicity but rather reflects the dysbalance of locally produced cytokines towards a proinflammatory profile. Hence, impairment of Sertoli cell function and subsequent breakdown of the blood-testis barrier appear to be important features of testicular inflammatory reactions [23]. With regard to the high prevalence of lymphocytic infiltrates, the induction of deleterious immune responses in the testis is probably not restricted to infectious agents, but a wide spectrum of etiological factors including neoplasia, chemicals, and physical trauma should be considered (fig. 1).

Interestingly, there is little evidence for a close relationship between testicular inflammatory reactions and formation of autoantibodies to spermatozoa, at least in humans. Whereas vasectomy or other injuries of the excurrent ducts are associated with a high prevalence of antisperm antibodies, only few patients with positive antibody titers were observed among those with a history of mumps orchitis [65, 66]. On the other hand, identification of immunodominant autoantigens by means of humoral immunity may help to improve diagnosis, classification and prognosis of testicular inflammatory reactions.

Conclusion and Outlook

It is now increasingly apparent that normal male reproductive function and the response to disease in the testis reflect different facets of the same regulatory environment. It encompasses complex interactions between testicular somatic cells (mainly Sertoli cells, peritubular cells and Leydig cells), resident

and circulating immune cells. Under normal conditions, immunoregulatory mediators dominate, protecting the spermatogenic cells by suppressing antigen-specific immunity and maintaining innate immunity, leading to an 'immunologically privileged' environment. Given that cytokines are regulators of normal testicular cell functions, and experience considerable upregulation during induction and amplification of cellular immune responses it is not surprising that dysregulation of the normal environment can activate the circulating immune cells, leading to a range of effects from temporary to permanent disturbance of spermatogenesis and steroidogenesis. From a clinical point of view, asymptomatic inflammatory reactions in the testis should not be neglected as an underlying reason or cofactor for male infertility. Further investigation may encourage the search for appropriate therapeutic strategies such as specific immunomodulation.

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The Role of Antisperm Antibodies during Fertilization and for Immunological Infertility

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Abstract

Antisperm antibodies (ASA) occur in men and in women. In the male they may cause an autoimmune disease, 'immune infertility'. In order to understand the functional relevance of ASA it is necessary to characterize the cognate antigens of ASA by different methods. In the literature, several immunologically characterized sperm proteins – as cognate antigens of naturally occurring ASA or of artificially produced antibodies – are quoted with respect to different sperm functions in the course of fertilization: cervix mucus penetration, zona binding, zona penetration, oolemma binding, and pronucleus formation. In a number of these functions, sperm proteins were already identified as cognate antigens of ASA. As practical consequences of the research on ASA-related sperm proteomics those ASA will be identified, which decrease male fertility by inhibiting sperm functions that are essential for fertilization.

Introduction

Antisperm antibodies (ASA) are an important cause of immunological infertility. The presence of ASA in males and females may play an important role in some cases of reduced fecundity. ASA occur in several human fluids, in the seminal plasma and bound to the sperm surface, in the blood serum of men and women, in oviduct fluid, cervical mucus, and in follicular fluid of women. ASA in the male may fulfill the criteria of an autoimmune disease. The knowledge about the specific mechanisms that elicit an autoimmune response and the active profile of ASA that leads to antibody-mediated infertility is poor.

ASA in men are assumed to occur mainly as a consequence of trauma to the blood-testis barrier, epididymis or vas deferens. They have also been

described as associated with inflammation, cryptorchidism, varicocele and surgical intervention in the genital organs. Often ASA appear to be of idiopathic origin. About 10% of infertile male partners have ASA in the seminal plasma or attached to the surface of spermatozoa. In addition, there is a high prevalence of these antibodies in men after vasectomy.

It seems likely that ASA binding to different antigens is related to the mode of antibody induction. Generally, when spermatozoa leak from the testis tubules, rete testis, epididymis or vas deferens, they come into contact with antigen-presenting cells, presumably macrophages, and a special immune response is induced. As we know, the antigenic properties of spermatozoa increase during passage through the epididymis because surface structures change. Thus, it is likely that the antigens detected by ASA from men with vasectomy but not by ASA from infertile men were related to the epididymal passage. If sperm leaks within the testis, it may be assumed that the ASA would show another antigen specificity.

Up to now different effects of ASA on sperm functions have been described. ASA affects sperm motility, the acrosome reaction (AR), and the penetration of cervical mucus by spermatozoa. Also, an inhibition of spermatozoa and zona pellucida (ZP) interaction and an interference with the sperm-egg fusion are described.

However, no proper methods exist to decide whether ASA of an individual man are of functional relevance, since only a limited number of sperm antigens may be associated with an influence on immunological infertility. It has to be clarified whether each ASA binding to a sperm surface antigen also influences sperm function. In the past, the clinical interest in ASA was hampered by the fact that a standardized and universally accepted assay for the detection of ASA was lacking. The characterization of the cognate antigens of ASA on sperm surface is the first step to understand the mechanism by which ASA may impair the sperm fertilization capacity. As clinical consequences of the research on ASA-related sperm proteomics those ASA will be identified which decrease male fertility by inhibiting essential sperm functions.

There are several methods to characterize the cognate proteins of ASA. Sperm proteins as cognate antigens of naturally occurring ASA are referred to below with respect to different sperm functions.

Tests for ASA

In the past, the clinical interest in ASA was hampered by the fact that a standardized and universally accepted assay for the detection of ASA was lacking [1]. Most centers used two tests for ASA detection, such as the mixed

antiglobulin reaction or the immunobead test, i.e. use of beads coated with antiglobulins. They are not able to identify specific antigens because of the relatively large 'labels' (erythrocytes, latex beads, polystyrol beads).

Also other test systems were used to detect ASA in human fluids. With immunofluorescence, only the detection of the immunglobulin class of the antibody concerned and the proportion of sperm-binding antibodies is possible, thus forming ASA 'titers'. The method is also unable to detect the number of antibody molecules or antigens involved in the binding. This concerns also flow cytometry, which was used for the determination of the number of sperm-binding ASA, giving comparable results of the immunobead test or mixed antiglobulin reaction test [2].

ELISAs and RIA-systems were hampered by the fact that the antigens were not biochemically defined and those relevant for the process of fertilization might not be contained in the antigen mixture [1]. There is an urgent need for tests that allow one to determine the antigenic moieties. ASA were shown to be directed against several different antigens, and each would be expected to have different effects on sperm functions. However, the discrimination between which antibodies are of clinical relevance and which are not is not possible by any of the test systems.

Analysis of Cognate Antigens of ASA and Influence on Sperm Function

There are several methods to characterize the cognate antigens of ASA. First, the localization of ASA binding on the surface of live spermatozoa of a healthy donor may be visualized by one of the methods of immune staining. If the results are compared to the sperm function in the patient from whom the ASA is derived, some insights into the functional relevance of ASA are possible.

Another method is the comparison of the localization of ASA binding to the binding of sperm-specific monoclonal antibodies (mAb) with a known cognate antigen. This also gives an approximate estimation of the localization of the antigen concerned.

A third approach to the analysis of antigen functionality is the analysis of proteins derived from DNA libraries of the testis. This approach, however, will lead to the identification of only part of ASA-inducing sperm antigens, since a number of sperm membrane antigens are secreted or altered during the epididymal passage.

The exact identification of the biochemical nature of the cognate antigens of ASA is possible only after a reliable separation of the sperm membrane

proteins and confirmation of their potency to bind ASA. This is achieved using two-dimensional electrophoresis of isolated membrane, blotting of the proteins to a PVDF membrane and immunostaining by binding of ASA. After this, the proteins isolated may be sequenced or analyzed using MALDI-MS (matrix-assisted laser desorption ionization-mass spectrometry) with subsequent peptide matching.

The comparison to known proteins in a protein database reveals the biochemical nature of antigens, giving rise to the identification of the proteomics of spermatozoa with respect to their function.

Agglutination

Investigation of the cognate antigens of agglutinating ASA, which were obtained from the blood serum of infertile women or were mAb raised in the mouse against human sperm proteins, identified YWK-II mAb with a cognate antigen immunlocalized in the equatorial sector of the human sperm head [3].

BE-20 is an epididymal-specific protein, whose ASA blocked the ability of human sperm to penetrate hamster eggs. The nucleotide sequence has high similarity to the proteinase inhibitors of the human epididymis. As a proteinase inhibitior it may assist in maintaining the integrity of acrosomes. rSMP-B, a sperm tail component, is recognized by ASA from the serum of infertile women. These antibodies may immobilize sperm and block their interaction with the egg. The gene was expressed only in spermatids. The human analogue (hSMP-1) is coded by the HSD-I gene, which is located on human chromosome 9, region p12-p13 [4].

BS-17 (calpastatin) was recognized over the acrosomal region and to a low extent on the tail. Antibodies blocked the penetration of hamster eggs, but neither the attachment to human ova nor inhibited sperm motility. The cDNA had 99.7% homology with the gene coding calpastatin. The gene was found to be transcribed only in spermatids.

HED-2 (zyxin) is a Sertoli cell component similar to EP-20. EP-20 was isolated from the caudal epididymis. Antibodies block motility and penetration of hamster eggs. Activation of a kinase might be the mechanism by which the HED-2 may trigger the signal transduction system of germ cells to undergo differentiation.

BS-63 cDNA contained repetitive sequence motifs, which are structural characteristic of nucleoporins. It is hypothesized that BS-63 is a testis-specific nucleoporin and possibly acts as a docking site and a cotransporter of Ran and transportin [5].

A mouse mAb A36 induced extensive 'tangled' sperm agglutination. The cDNAs encoding its cognate antigen displayed >99% homology to glucose phosphate isomerase (GPI). The mAb A36 cognate sperm surface antigen is a GPI-like protein involved in sperm agglutination [6]. An mAb that immobilized human sperm was shown to react also with seminal plasma, indicating that the cognate antigen on the sperm surfaced might stem from the epididymis [7].

Sperm agglutination is closely related to immune contraception. Several studies describe antibodies to sperm proteins, which lead to intense sperm agglutination, being candidates for immune contraception [8]. A polyclonal antibody, raised against a 16-kDa human sperm protein identified by an mAb to human sperm, showed extensive agglutination both in mouse and human spermatozoa. Passive immunization of female mice with this antibody caused 67% reduction in fertility [9]. A recombinant single-chain variable fragment (scFv) antibody binds to a tissue-specific carbohydrate epitope located on human sperm agglutination antigen-1, the sperm glycoform of CD52. The recombinant antisperm antibody (RASA) aggregated human spermatozoa in a tangled (head-to-head, head-to-tail, tail-to-tail) pattern of agglutination [10].

Motility

Since it is likely that ASA only bind to antigens of sperm membranes, while subcellular structures are not reached by ASA in the living cell, it is difficult to explain how ASA interferes with sperm motility. It may be speculated that the function of proteins with intra- and extramembranaceous parts may be altered by the ASA concerned.

Sperm motility parameters determined by computer-assisted semen analysis showed no differences between samples containing ASA and those free of ASA [11]. Various tubulins are involved in the functional organization of the mammalian sperm flagellum and head. mAb directed against epitopes on the C-terminal end of neuron-specific class III β-tubulin stained the flagella [12]. Another family of filament-forming proteins are the tektins that are coassembled with tubulins to form ciliary and flagellar microtubules. Tekt1 is one of several tektins to participate in the nucleation of the flagellar axoneme of mature spermatozoa, perhaps being required to assemble the basal body [13]. Other results suggested that flagellar movement of sperm is also modulated by proteasomes, which regulate the activity of outer dynein arm by cAMP-dependent phosphorylation of the 22-kDa dynein light chain [14].

Complement regulatory proteins such as C1-INH, CD55, CD46, and CD59 were expressed on sperms [15]. IgG fraction of antibodies to these proteins significantly reduced sperm motility in general and other motion parameters.

Chloride/bicarbonate (Cl^-/HCO_3^-) exchangers, a family of proteins [anion exchanger (AE) gene family] that regulate many vital cellular processes such as intracellular pH, cell volume, and Cl^- concentration, are also involved in the regulation of sperm motility. The AE2 isoform of a sperm cell anion exchanger of this gene family was identified in sperm [16].

Cervix Mucus Penetration

Impairment of sperm penetration into the cervical mucus appears – in contrast to the other antibody effects described – to be a consequence of the activation of the complement cascade by immunoglobulins attached to the sperm surface, at the end of which cell lysis and initiation of the phagocytotic process may take place. The complement-induced cell lysis depends on the immunglobulin class; IgM (which is not present in semen) is far more effective than IgG, while some IgA subclasses are unable to interact with the early complement components.

Complement-activating ASA may be effective only in the mucus, because the seminal plasma contains complement inhibitors. During their residence in the cervical mucus, spermatozoa are exposed to complement activity. Since levels of complement are lower in cervical mucus than in serum, it may take longer for sperm immobilization to occur. This is the rationale for performing postcoital tests only after 6–7 h.

The impairment of the cervical mucus penetrating ability of ASA of the IgA class appears to be mediated through the Fc portion of the IgA. It was shown that after a degradation of IgA bound to the sperm surface by an IgA protease from *Neisseria gonorrhoeae* mucus permeation was no longer inhibited [17].

Acrosome Reaction

A number of spontaneously occurring ASA was shown to enhance the number of acrosome-reacted sperm, but none of them was able to inhibit AR in vitro [18]. An antiactin mAb significantly inhibited the ZP-induced AR (equivalent to cytochalasins), the ionophore A23187-induced AR and hyperactivation of sperm [19].

TSA-1 was specifically expressed only in the human testis. Antibodies produced against the computer-generated translated protein bind to acrosomal, equatorial, midpiece, and tail regions of human spermatozoa and caused a significant and concentration-dependent inhibition of human sperm AR [20]. When seminal plasma samples containing ASA or spermatozoa loaded with ASA were adsorbed

with fertilization antigen-1, the percentage of immunobead-free swimming sperm increased on an average by 50% [21]. The rate of spermatozoa undergoing AR as induced by the calcium ionophore A23187 showed an improvement in 78% of the sperm samples after fertilization antigen-1 (FA-1) adsorption.

Antisera against a porcine liver endomembrane progesterone-binding protein inhibited the progesterone-initiated AR of human sperm [22]. Indirect immunofluorescence studies detected antigens in the sperm head that moved during capacitation from a posterior head region to a mid-head region. This observation suggested that a sperm protein with at least partial homology to the liver endomembrane progesterone-binding protein is a progesterone receptor on the sperm plasma membrane.

The two intra-acrosomal molecules acrin 1 (MN7) and acrin 2 (MC41) are essential for distinct events before sperm penetration of the ZP in mice. The mAb mMN7 prevented completion of acrosomal matrix dispersal, whereas mMC41 did not affect the AR. mMC41 appeared to inhibit secondary binding or some biochemical steps on the ZP after the AR but before penetration of the ZP [23].

The AR involves different activities requiring Ca. The elevation of intracellular calcium and bicarbonate concentrations stimulates adenyl cyclases to produce cyclic AMP, which activates phosphorylation of certain proteins by the protein kinase A (PKA). For a continuation of the AR, further activation of cyclic AMP/PKA and protein kinase C (PKC) is necessary. PKC opens a calcium channel in the plasma membrane. PKA together with inositol-trisphosphate-activate calcium channels in the outer acrosomal membrane leads to an increase in cytosolic calcium [24].

Calpastatin, a 17.5-kDa protein, is an integral part of the acrosomal cytoplasm. Using polyclonal antibodies to calpastatin, immunstaining was seen over the acrosomal region and slightly on the tail. The gene was found to be transcribed only in spermatids. The inhibition of calpastatin led to a premature AR [3]. Calpastatin binds calpain, a Ca-dependent cysteine endopeptidase. This protease system may be functional in (cynomolgus macaque) sperm during capacitation, the AR, or both [25]. Antibodies to calpains bound to the region between the plasma membrane and the outer acrosomal membrane of sperm. After the AR, all of the anticalpain antibodies labelled the acrosomal shroud presenting acrosomal contents, which suggests that calpains are located throughout the cytoplasmic area between the two outer sperm membranes.

Two forms of clusterin have been detected in the human male reproductive tract: the conventional heterodimeric form and a novel acrosomal form. Specific immunogold labeling showed the antigen present on the surface mainly of the acrosomal contents exposed by the loss of the plasmalemma and outer acrosomal membrane [26].

A neuronal glycine receptor/Cl^- channel (GlyR) was detected on the plasma membrane of mammalian sperm. Pharmacological studies suggested that this receptor/channel is important for initiation of AR by the zona pellucida. An mAb against GlyR completely blocked ZP initiation of AR in normal mouse sperm. These findings indicate that sperm GlyR plays an essential role in the AR as initiated by the ZP [27].

Zona Binding and Zona Penetration

The binding of the sperm to the ZP occurs via specific receptors localized over the head region of the spermatozoa. ZP binds at two different receptors in the sperm membrane. One is a G_i-coupled receptor that activates β_1-phoshporlipase C, the other one is a tyrosine kinase receptor coupled to γ-phospholipase C [24].

Three nucleotide sequences encoding SP22 were identified, a protein originally identified in detergent extracts of cauda epididymal sperm, from a rat testis cDNA library. There was evidence that SP22 was a member of a highly conserved and widely expressed gene family. An antibody to SP22 peptide bound to the anterior-ventral surface of the equatorial segment of the sperm head. Although no conclusive function has been attributed to any members of the SP22 gene family, the localization of SP22 over a discrete region of the sperm head suggested a role in sperm-egg interactions [28].

A protein (Ag 1F10) was described as composed of a single peptide chain. The zona-binding activity of spermatozoa preincubated in the presence of an mAb to this protein was significantly inhibited by human in vitro fertilization systems. The authors assumed that the protein bearing the epitope recognized by the mAb might be one of the molecules with receptor function in sperm-ZP interaction [29]. A dodecamer sequence, designated as YLP(12), is involved in sperm-ZP recognition/binding. The peptide sequence was localized on the acrosomal region of the human sperm cell [30].

Patients expressed ASA in their sera that bound to the sperm surface, most specifically the head region, and that reduced ZP tight binding of spermatozoa as assessed by the hemizona assay. One of the possible antigens involved in zona penetration is PH-20, a glycerol-phosphatidylinositol-linked hyaluronidase. In the guinea pig, two regions of this enzyme were highly immunogenic. These regions are accessible on the sperm surface when native gpPH-20 is in solution or anchored on sperm surface. Since PH-20 is present in many species and also in humans, it may be a cognate antigen of ASA in humans [31]. In mice, a sperm antigen designated as FA-1 was identified [32]. The cDNA was cloned and sequenced and was able to translate a protein which specifically reacted with zona protein 3 (ZP3) of oocyte ZP. When polyclonal antibodies were

generated, they completely blocked sperm-ZP interaction in mice. Similar results were found in the human system.

Oolemma Binding

A 20-kDa glycoprotein (GP20) was isolated from human sperm [33]. An anti-gp20 antibody intensely stained the head and midpiece; however, on acrosome-reacted spermatozoa the antibody binding was restricted to a small band in the equatorial region. The antibody did not only bind to sperm precursor cells in the testis but also to epididymal epithelial cells. Thus, it seems to be a protein added to the sperm membrane in the epididymis and represents one of the sperm-coating antigens. Anti-gp20 exerted a blocking effect in a test for sperm penetration of zona-free hamster eggs. This concerns also an abundant epididymal gene product that has been identified as lymphocyte surface antigen CD52.

A human sperm protein was described, which bound *D*-mannose coupled to albumin (DMA) in the presence of cations and a neutral pH. The binding of human spermatozoa to zona-free hamster eggs was reduced by DMA in a dose-dependent manner, suggesting that DMA-binding sites in human spermatozoa are involved in sperm-egg fusion [34]. Cryopreserved rooster sperm was found to bind to the perivitelline membrane of a chicken egg, when a protein isolated from the supernatant of cryopreserved rooster sperm was added. The partial purification of this activity demonstrated a fragment of prosaposin, which is also present in human semen. This observation may also be of relevance for human fertilization and its impairment by ASA [35].

SP-10 is a sperm intra-acrosomal protein, which is produced specifically in the testis, but expressed in human spermatozoa only after AR. An mAb to this protein inhibited sperm-oolemma binding in the zona-free hamster egg penetration test, but it did not inhibit sperm-zona binding in the hemizona assay. Human SP-10 was found to mediate sperm-oolemma binding in a β_1 integrin-independent manner, but not sperm-zona binding [36]. A CD46 (membrane cofactor protein of complement) isoform is found on human spermatozoa, which was associated with the sperm-egg interaction. The expression of CD46 in other cells confers resistance to complement-mediated injury.

Pronucleus Formation

At gamete fusion the sperm tail is incorporated into the ooplasm, and the centriolar region forms the sperm aster. While the sperm head is decondensing, the aster guides the female pronucleus towards the male pronucleus. ASA against

proteins of the centrioles may be responsible for mitotic arrest [37]. The human nuclear autoantigenic sperm protein (NASP) is a testicular histone-binding protein to which most vasectomized men develop autoantibodies. In a study recombinant deletion mutants spanning the entire protein coding sequence were screened with sera of vasectomy patients [38].

An unanswered question is that of sperm apoptosis. Several proteins of the signal transduction pathways of apoptosis are present on the sperm surface. It is questionable, however, whether these proteins are functionally active. The presence of apoptotic proteins in ejaculated spermatozoa may be linked to defects in cytoplasmic remodeling during the later stages of spermatogenesis. ASA binding to the inactive form of caspase-3 as a cognate antigen was demonstrated [39]. The pathophysiologic significance of these ASA is still unclear.

Treatment Options

The identification of functionally relevant antigens is a prerequisite for treatment options. At present, no antibody-specific treatment of autoimmune diseases is possible, but the treatment is based on the suppression of antibody production in general. Increasingly, however, the use of mAb in autoimmune diseases is described. It can be speculated that this procedure may also be adapted to the treatment of autoimmune infertility. At present, performing intracytoplasmic sperm injection (ICSI) is the only promising treatment procedure to overcome ASA effects inhibiting fertilization. The analysis of the cognate antigens of ASA involved in the process of fertilization is important from another point of view: it improves the identification of immunogenic proteins which are candidates for immune contraception, i.e. which allow the artificial induction of antibodies in males or females inhibiting fertilization.

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Table 1. Immune cells in the normal adult testis [5, 8, 9]

Cell type	Human	Rat
Macrophages	++	++
Mast cells	+	(+)
Lymphocytes	(+)	+[a]
Natural killer cells	?	(+)
Dendritic cells	?	?
Granulocytes	–	–

[a]CD8+ > CD4+ T cells, no B cells.

cell steroidogenesis in the adult testis [14]. In mice lacking colony-stimulating factor-1, reduced numbers of testicular macrophages result in impaired spermatogenesis as a consequence of dramatically reduced testosterone levels [16]. Beyond their impact on testis-specific functions, macrophages in the testis have to be considered as potential effector cells in the first line of host defense, i.e. activating innate immune responses and thus inflammation. Notably, testicular macrophages have been shown to express major histocompatibility complex class II (MHC II) molecules essential for antigen presentation to CD4+ T cells [4, 5, 9, 15]. However, the ability of freshly isolated rat testicular macrophages to release proinflammatory cytokines such as interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α) is reduced in comparison with macrophages of other origin [17, 18]. Available data suggest that resident macrophages in the normal adult testis mainly exert anti-inflammatory activities [14].

Similar to macrophages, mast cells seem to be involved in the complex local regulation of testicular functions [5]. Mast cells are known to play an important role in both innate immunity as well as specific acquired immune responses and are capable to release a wide variety of inflammatory mediators such as tryptase and other proteases, histamine, leukotrienes, prostaglandins, and cytokines [19]. In the adult human testis, mast cells can be found in the interstitium, within the lamina propria of seminiferous tubules, and in the tunica albuginea [11].

Approximately 15% of the immune cells in the normal adult rat testis were shown to be lymphocytes [5]. Most of these lymphocytes expressed T cell markers with a predominance of CD8+ T cells, whereas B cells were not detectable. Concerning the human testis, studies are scarce and only qualitative data are available. Immunocytochemistry revealed few or no lymphocytes in the normal peripheral testicular tissue, whereas considerable numbers of T cells were detectable within the lining epithelium (CD8+ > CD4+) and in the

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Target Antigens for Sperm-Immobilizing Antibodies Found in Infertile Women

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Abstract

Complement-dependent sperm-immobilizing antibodies (SI-Abs) have frequently been detected in the sera of infertile women. Analyses using monoclonal antibodies as well as patients' antisperm antibodies have shown that the carbohydrate moieties of sperm and seminal plasma are major epitopes of these antibodies. In the present study, we characterized the antigen molecules recognized by human monoclonal SI-Ab (MAb H6-3C4) derived from a patient with strong SI-Ab activity. Immunofluorescent staining showed that MAb H6-3C4 reacted with ejaculated human sperm and the epididymis but not with the testes and other somatic organs including the spleen. In Western blot analysis, MAb H6-3C4 reacted to antigen molecules of Mr 15–27 kDa present in the methanol-chloroform extracts of either ejaculated sperm or seminal plasma. Amino acid sequence analysis of the reactive molecule revealed that the amino acid sequence was identical to the core peptide of CD52, a GPI-anchored protein on lymphocytes. Furthermore, the epitope for MAb H6-3C4 was found to be the specific N-linked carbohydrate for the male reproductive tract. These results suggest that SI-Abs in some infertile women target a sperm-specific carbohydrate antigen on CD52 molecules which are secreted from the epididymis and coat the sperm surface.

Introduction

Antisperm antibodies detected by sperm immobilization test are frequently detected in unexplained infertile women [1, 2]. When the sperm-immobilizing antibodies (SI-Abs) are present in serum, they are also found in the cervical mucus, the peritoneal fluid and even in the follicular fluid, and patients with a high titer of SI-Abs find it very hard to conceive a child [3, 4]. As a possible role in infertility our clinical studies showed that SI-Abs impaired sperm passage in

the female reproductive tracts from the cervix through the fallopian tubes, and fertilization at the level of sperm binding to the zona pellucida [5, 6]. Although analyses using patients' antisperm antibodies have shown that the carbohydrate moieties of sperm and seminal plasma are major antigens of these antibodies [7, 8], the antigenic epitope relevant to SI-Abs has not yet been fully characterized. Identification of the epitopes recognized by SI-Abs is important not only for understanding the mechanism of immunological infertility but also for developing a means of treatment for infertility due to SI-Abs.

Characterization of Monoclonal Antibodies Showing SI Activity

For this purpose, a number of monoclonal antibodies showing SI activity including human antibodies were generated [9–11].

Previously, using peripheral B lymphocytes from an infertile woman, a stable human-mouse heterohybridoma, H6-3C4, producing a human monoclonal antibody with high titers of SI activity was established in our laboratory [12]. In this article we describe the characterizations of three monoclonal antibodies (MAb H6-3C4, 1G12 and campath-1) inducing agglutination and immobilization in human sperm. 1G12 is a monoclonal antibody secreted by mouse hybridoma generated with human sperm membrane extracts [13]. Campath-1 is known to be a monoclonal antibody recognizing CD52 on virtually all lymphocytes and cross-reactive to mature human sperm [14, 15]. MAb H6-3C4 reacts exclusively to sperm, while the other two monoclonal antibodies also react to other somatic tissues.

Indirect immunofluorescent analysis revealed that the monoclonal antibodies stained over the whole sperm, especially the postacrosomal and tail regions (fig. 1). It seemed that the antigens recognized by these monoclonal antibodies are similarly distributed on the sperm surface.

In SDS-PAGE analysis, MAb H6-3C4 reacted to antigen molecules of Mr 15–27 kDa present in the lipid extract fraction of either ejaculated sperm or seminal plasma. Amino acid sequence analysis of the immunocomplex formed by the antigen molecule with MAb H6-3C4 revealed that the antigen is the same core peptide composed of 11 amino acids as that of CD52 [16, 17]. CD52 is a GPI-anchored protein recognized by campath-1 and present on all lymphocytes. Since MAb H6-3C4 did not react to lymphocytes as mentioned above, further investigation was necessary.

To find the epitopes of the antibodies, sperm extracts were subjected to high-resolution two-dimensional polyacrylamide gel electrophoresis with the first dimension in a pH 2–4 range and then Western blot analysis (fig. 2). MAb H6-3C4 and 1G12 yielded staining patterns similar to those with campath-1,

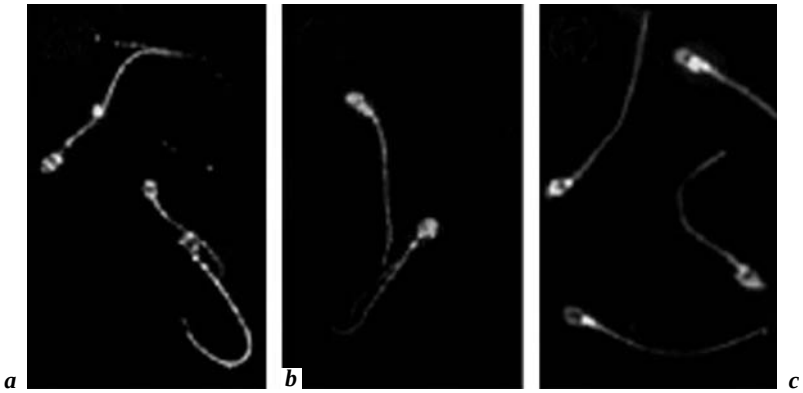

Fig. 1. Immunofluorescent staining of formalin-fixed sperm with monoclonal and polyclonal antibodies. MAb H6-3C4 (***a***), 1G12 (***b***), campath-1 (***c***) strongly stained the whole sperm, especially the postacrosomal and tail regions.

reacting to the extremely acidic components. With all the antibodies used, positive stainings appeared in the region of Mr 25–35 as well as 17–25 kDa, both of which showed heterogeneous patterns comprising different pH components (fig. 2a–c). Collectively, these results suggested that MAb H6-3C4 and 1G12 recognized CD52 similarly to campath-1. Further precise examination such as carbohydrate analysis was needed to clarify this contradiction.

For carbohydrate analysis, CD52 extracted from sperm was subjected to enzyme treatment. Endo-β-glycosidase F was employed to remove the N-linked carbohydrate. The reaction of MAb H6-3C4 with sperm extract disappeared after treatment with N-glycosidase F (fig. 2d). From this result we confirmed that the epitope for MAb H6-3C4 is present in the N-linked carbohydrate moiety of CD52 reported by Diekman et al. [18]. The present findings also indicate that SI-Abs in some infertile women are produced against a sperm-specific carbohydrate antigen on the CD52 molecule which is secreted from the epididymis and coats the sperm surface. Indeed, the occurrence of specific types of carbohydrate has been found in the male reproductive tract CD52 [19].

In contrast, 1G12 and campath-1 still showed positive reactions after N-glycosidase F treatment, with a shift of staining to lower Mr regions (fig. 2e, f) and persisting pH heterogeneity. 1G12 reacted with 6 spots at different pH, while campath-1 reacted with three different spots. This indicates that the epitope for 1G12 is not identical to that of campath-1. In addition, a further mild alkaline treatment to remove O-linked carbohydrates resulted in a decrease in the number of spots reacting to 1G12 and campath-1 reacting to only one at Mr 13 kDa and pH 3.5 [17]. These results suggested that male reproductive tract CD52 contains

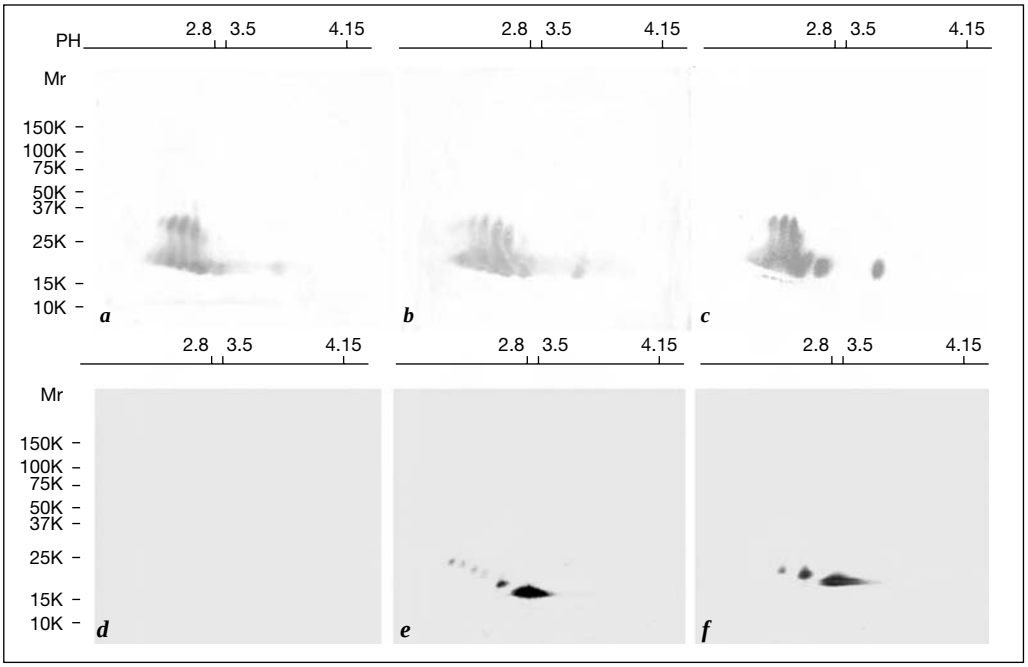

Fig. 2. Two-dimensional PAGE and Western blot analysis of sperm extract with monoclonal antibodies before and after treatment with N-glycosidase F. The sperm extracts were separated by two-dimensional PAGE, blotted onto a PVDF membrane and probed with monoclonal antibodies. ***a***, ***d*** MAb H6-3C4. ***b***, ***e*** 1G12. ***c***, ***f*** Campath-1. Three monoclonal antibodies showed a similar polymorphic pattern before removal of N-linked carbohydrate (***a***–***c***). The pattern changed after removal of N-linked carbohydrate in each monoclonal antibody (***d***–***f***).

O-linked carbohydrates, differing from lymphocyte CD52 that does not contain this type of carbohydrate. A hypothetical structure of male reproductive CD52 is shown in figure 3.

Biological Function of CD52

The biological function of mrtCD52 in mature sperm is not well understood. The glycosylated molecules including N-linked and O-linked carbohydrates make the CD52 molecule highly negatively charged. Not only N-linked carbohydrate but also O-linked carbohydrate possibly contribute to the heterogeneous negative charge of mrtCD52 [20]. Such a structure may prevent lymphocytes and

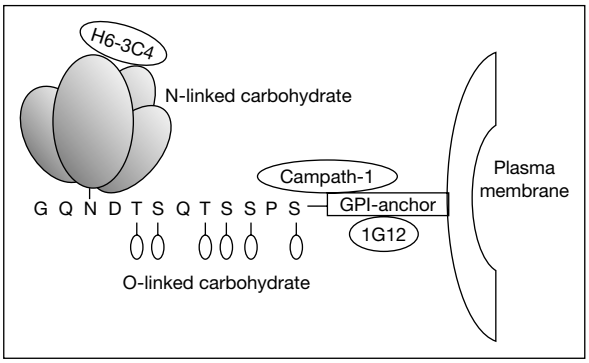

Fig. 3. Hypothetical structure of male reproductive tract CD52. The male reproductive tract CD52 molecule is composed of the 12-amino acid peptide, N-linked and O-linked carbohydrates, and a GPI anchor portion inserted in the plasma membrane. This structure is quoted from Hasegawa et al. [17]. The amino acid sequence of core peptide is shown in one-letter cords for amino acids. Carbohydrate chains are shown by ovals.

sperm from autoagglutination and nonspecific adherence to tissues as previously proposed [21].

Considering that the monoclonal antibodies targeted to mrtCD52 exhibit extremely strong SI activity with complement, it is an attractive hypothesis that CD52 possesses the function to suppress complement activity. Campath-1 recognizing lymphocyte CD52 also has been shown to induce complement-dependent cell lysis [22]. The female genital tract is subject to frequent infection with various pathogens including sexually transmitted bacteria and viruses. However, its antibody-producing ability is not so high compared to other mucosal tissues [23]. Innate immunological systems such as complement are thought to serve mainly as a host defense mechanism. Functionally active complement exists in the female genital tract [24] and follicular fluid [25].

Recently, complement-regulatory proteins such as C1-INH, CD55, CD46 and CD59 were found on the surface of spermatozoa [26], and CD55 and CD59 were shown to be GPI anchor proteins [27]. Collectively, it is speculated that GPI anchoring and complement-regulatory proteins seem to play a role in protecting sperm from complement attack during transportation to the fertilization site.

Conclusion

The mechanism by which mrtCD52 induces antibodies in the female genital tract is not known. Normally the N-linked carbohydrate of the CD52

molecule on sperm is not recognized by immunocompetent cells as an antigen, as shown by the fact that the frequency of detection of SI-Abs in infertile women is not particularly high. Local pathogenic conditions such as genital infections may contribute to enhance the immunological responses. Spermatozoa falling into the abdominal cavity would easily be recognized by macrophages as a foreign antigen. Alternatively, it is possible that some molecular change of mrtCD52 caused by genetic alterations may enhance immunological responses in the female genital tract. In addition, several immunosuppressive factors have been reported in seminal plasma [28–30]. The deficiency of these factors in seminal plasma may enhance the production of sperm antibodies in female. Although mrtCD52 is not the only etiological sperm antigen for infertility, knowledge about its physiological function and molecular structure is helpful for understanding the mechanism of the production of antisperm antibodies and for developing means of treatment for infertility.

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Cytokines and Implantation

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Abstract

This review introduces the field of cytokines and implantation and then recalls the specialized role of the uterus and the notion of the 'implantation window'. The role of inflammatory and angiogenic cytokines is presented, as well as the involvement of cytokines such as τ-interferons in corpus luteum maintenance in non-chorionic gonadotrophin-producing species. τ-Interferons are reviewed, before dealing with the more in depth analysis of cytokine networks in the pre- and peri-implantation uterus. The emerging involvement of cytokines in controlling uterine vascularization angiogenesis is reviewed, with emphasis on NK activating factors.

Introduction

One of the most important discoveries in the field of reproductive immunology is that cytokines are involved directly in reproductive functions. This stems from the original immunotrophic theory [1], and the subsequent demonstration that T cell-secreted cytokines were growth factors for trophoblasts [2, 3]. The demonstration that these cytokines could positively or negatively affect early embryo resorption in a murine spontaneous abortion model [4] was completed by the observation by the group of Pollard [5] that the colony-stimulating factor 1 (CSF-1) was expressed very early in the pre- and peri-implantation uterus, and that the levels increased throughout pregnancy. Though the sterility observed in CSF-1-negative mice [6] was later on shown to be attributable more to the male than the female [7], those observations triggered studies which were devoted to the systematic search for the presence and involvement of cytokines in the preimplantation uterus, and later on in or on the embryo itself. As Tom Wegmann

said: we are familiar with the concept that the embryo is immersed in a sea of cytokines, and also … that it does produce cytokines itself!

The (Still Mysterious!) Role of the Uterus

For the embryo to implant there must be a perfect synchronization between the uterus and the embryo itself. Too early means the uterus has not undergone a proper decidual transformation, and the embryo will not implant. Too late means the uterus will either be in a state where it prepares itself to resume the cycle, or it will even more prosaically reject the blastocyst.

This defines the implantation window, whose discovery in the rabbit by the late Charles Thibault [8] was an important step in reproduction, and whose delineation in humans [see 9] was one of the clues whose discovery permitted Edwards and Steptoes to perform the first successful IVF-ET, leading to the birth of the first IVF baby, Louise Brown. This was achieved against all odds including those of Thibault himself in an SFEF meeting a few weeks before this very birth of Louise Brown.

One of the mysteries of the implantation window is that it *cannot* be seen outside the uterus [9, 10]. Indeed, extrauterine pregnancies can occur almost everywhere, and the few cases per year of term abdominal pregnancies (which, if the 'pseudo-cesarean' delivery is performed well by the surgeon, results in normal weight and healthy babies!) testify that there is no special organ requirement linked to an immunological component for implantation to occur outside the uterus.

The uterus is, therefore, the only organ which will not accept an embryo (except in the so-called implantation window) and is thus outside, this time period totally refractory to implantation, and this remains as much a mystery as there are not many studies on cytokine localization in extrauterine pregnancies. These are, nevertheless, badly needed, since there is, for example, consensus [see below and 10, 11] that NK migration/accumulation in the immediate pre- and peri-implantation period is one of the characteristics of the immediate preadhesion and peri-implantation uterus, while according to most (but not all) studies (only one of them has been published in a traceable form on Medline), tubal pregnancy implantation sites are devoid of such accumulation [12]. Indeed, when scrutinizing papers published on artificial tubal and abdominal pregnancies in rodents, we found no report of such cell trafficking, be it in female or male (!) recipients. Nevertheless, a certain number of studies have been devoted to the delineation of differences between tubal and uterine pregnancies, by estimating cytokine levels in either the serum or on tubal fragments obtained after surgery. Two cytokines have been studied most frequently, VEGF [13–15] and

LIF [16, 17], but so have others such as IL-18 and TNF [18]. It is only fair to say that the results are rather controversial and thus not fully conclusive, though suggestive indeed of the two cytokines.

The caveat for such studies is that the kinetics might be different from a uterine implantation site, and that NK might already have accumulated and receded; at the same time cytokine profiles might have been altered, since tubal pregnancies are indeed diagnosed when implantation has already occurred, and invasion of tubal arteries transformed the process into a surgical emergency, with all the criticism that can be raised against such studies as far as cellular kinetics are concerned, since the embryo implantation site is most often observed once the initial steps of implantation are completed (see above).

Nevertheless, it follows that the precise function that a preimplantation uterus should fulfil is in fact far from being well understood. However, it seems that there is consensus to say it depends on transformation of the decidual stroma for acquiring nutritive properties, expressing adhesion molecules, and perhaps, transformation of the matrix to render it more sensitive to enzymes such as proteases permitting both embryo adhesion and subsequent invasion.

Indeed, it should be recalled that the embryo also expresses a certain number of cytokines, acting in a paracrine or autocrine fashion, which will thus either act on the embryo itself, or on the uterus proper. So, one of the first things which has been looked for is the production of such factors by the embryo as a correlate of implantation.

Embryonic Cytokines and Implantation?

The search has been going on since the early description by Morton et al. [20–22] of an early pregnancy factor (EPF) [23–29] to which immunosuppressive properties had been attributed in a cascade fashion akin to the I-J+ factors from Ts of an infamous reputation. Indeed, there have been several papers each claiming to have achieved 'purifications to homogeneity' of EPF, sometimes by the very same people with conflicting results. The (last) claim is that the material has finally been successfully identified as a chaperonin 10 extracellular homolog, albeit the authors themselves do admit one could still debate about its dentity [29]. Indeed, others have identified it as an Fc receptor-like molecule [30]. So far, despite claims of effects in murine models of autoimmunity [31, 32], EPF has failed to gain wide acceptance in immunology, especially since assays in animals on a large scale at the Institut National de la Recherche Agronomique (INRA) failed to reproduce even the predictive value of the original detection of 'immunosuppression' by the rosette inhibition test [Thibault, pers. commun.].

There has also been a report by Mayumi et al. [33] of a suppressor cell-inducing factor in the supernatants of murine blastocysts, but this molecule has never been confirmed. As for the report by Daya and Clark [34] of an immuno-suppressive factor in human blastocyst supernatants, we failed to reproduce it ourselves [35] and it was later on attributed to a spermine contaminant. Better defined apparently is the preimplantation factor, but a series of patients cover the molecule(s) and peptides derived from it, so that the precise nature of this molecule cannot be described in this review, and we will have to wait till further publications of the structures and effects of the materials become available [36, 37].

As far as 'known' cytokines are concerned, their production by the embryo is well documented and is indeed stage specific, as shown very well for example in Sharkey et al. [38]. This has been explored in more detail: in rodents, artificial models of delayed implantation have been developed, where the embryo does not attach until a progesterone surge occurs in the mother. This is also seen in several species, such as the mink and marmoset, where the embryo conceived during the autumn season does not implant till spring and remains floating in the uterine horn till spring, when a surge of progesterone and subsequent LIF production by the maternal uterus (see below) permits implantation.

Several cytokines are expressed in early human embryos [39, 40], some as early as at the 1st cell stage; there are inflammatory cytokines such as IL-1 [37]. The delayed implantation system in the rat permits studies of hormonal influences in a specifically hormone-deprived environment (hormones and hormone-like materials are often present in culture media). In this system, their role has been demonstrated, since the so-called 'dormant' blastocyts do not express inflammatory cytokines, whereas activation by a hormonal surge results in their immediate production as early as at the 4-cell stage [41].

Thus, the temptation has been great, and the search has been going on, to try to correlate the production of such cytokines by the embryo with successful implantation. Here again the search has yielded conflicting data, and so far no conclusive consensus evidence has been obtained [42–48]. In some cases the coculture systems used make it difficult, especially for LIF (for example with Vero cells), to identify the precise cellular source of the cytokine (we would like to mention here that human cell lines have been described as putative replacement of Vero cells in this respect [49]).

It should be mentioned, however, albeit this is not dealing with cytokines, that there are two recent reports claiming a correlation of sHLA-G production by human preimplantation embryo and implantation rates. In the first of these reports, only sHLA-G-producing embryos can implant [50] (but all the sHLA-G-positive ones do not do so; therefore, it is a necessary, but not sufficient, requirement). In the second report, there is a strong predictive correlation only [51].

Maintenance of the Corpus luteum in Ovine and Bovine Species: The τ-Interferons

In humans, and in fact in mammals, pregnancy depends on the maintenance of the corpus luteum. In primates, the luteolysis is blocked because the early embryo secretes a hormone (not a cytokine, hence its absence in this review), human chorionic gonadotrophin (hCG). There are many species where chorionic gonadotrophins are involved (failure of equine CG appearance in mare's blood is the first sign of a failed pregnancy in the equid donkey in the horse embryo transfer system) [52]. In humans, the presence of hCG in the urine of women is one of the earliest characteristics of pregnancy (hence almost all self-pregnancy tests now freely available at any drugstore are anti-hCG ELISAs).

But in ovine and bovine species, there are no such chorionic gonadotrophin hormones. The search for an equivalent material led to isolation, sequencing and cloning by the INRA groups under the direction of Jacques Martal of a material named trophoblastin, and renamed, in a typically Anglo-Saxon way, by English-speaking scientists ovine trophoblast protein (oTP).

To the surprise of Martal and colleagues [53–55], sequencing and cloning of oTP revealed a molecule closely related to interferon-α. Indeed, it was demonstrated that the molecule defined a new class of interferons, the τ one, highly conserved across species [56–60].

These molecules (in variance with γ-interferon – which are abortifacient at high doses in rodents and human) are neither cytostatic nor cytotoxic for the trophoblasts, which secrete them. In variance also with interferon-γ and even interferon-α, which both require a cell activation signal, their secretion is programmed during trophoblast development, and thus it occurs without an external signal, but under the developmental program, and this only during the pre- and peri-implantation period at the trophoblast/trophectoderm 'elongation' stage. The secretion rate is a much higher than what is seen for other interferons, even those in the case of γ-interferon in activated lymphocytes [56–58].

The molecule is a bona fide interferon with classical cytostatic properties on activated T lymphocytes [59, 60] with variations according to isoforms [62]. Thus, recombinant oTP was tested as an early signal/component of tolerance to the fetus, which proved to be also true when tested in a murine model of GVH/HVG tolerance [63]. It also has a series of immunoregulatory activities, and indeed in a murine model of spontaneous abortion, it prevents abortion and increases implantation rates by signalling Th2 cells and Th2-like cells to secrete high amounts of Th2 cytokines. Thus, it explains why the maternal immune system is biased towards a Th2 profile before maternal immune recognition of the fetus [64]. As such, it has been tested with success in a murine model of

allergy [65], and more importantly, by oral administration, also with success in the treatment of autoimmune encephalopathy [66]. It is also endowed with excellent antiviral properties in animals [67] as well as in humans including against HIV [human studies are being conducted at the CEA (Commissariat à l'Energie Atomique), France, in cooperation with INRA] [68].

Besides such therapeutic potentials, τ-interferons were among of the first truly 'immunoendocrine' cytokines, a fundamental quantum leap discovery. Their actions are not limited to the corpus luteum and the Th2 profile, but have several targets in the uterus [for reviews, see 55–59]. The most recent examples are the attractive possibility of effects on chemokines (and thus possibly on the recruitment of NK cells), but the attracted lymphocytes have not yet been fully characterized [69], the regulation (as expected) of MHC antigen expression [69], and most interestingly the regulation of secretion in the reproductive tract of cytokines such as GM-CSF and MIF [71, 72]. Finally, it could have direct effects on a uterine epithelium [73], and possibly even act in an autocrine way on the embryo itself [74]. It is important to stress here that in vitro and in vivo expression kinetics of the embryo differ [75]. This is very important, since there is accumulating evidence that τ-interferons could be very important in the adhesion process, as early as at the blastocyst stage [76].

The differential effects of interferon-τ and γ, apparently using the same receptors and the same transduction pathway [77–78], were puzzling. A clue might recently have been found by the group of Roberts [79]. In a series of *very* elegant experiments, this group seems to have demonstrated that IFNAR2, one of the two subunits of the receptor, might interact with a stress-activated protein kinase (SAPK)-interacting protein 1 (ovSin1) as a protein that bound constitutively through its own carboxyl terminus to the receptor. ovSin1 is a little-studied 522-amino-acid-long polypeptide (Mr 59,200), which is highly conserved across vertebrates, but which has identifiable orthologs in Drosophila and yeast, and is expressed ubiquitously in mammals, although at low abundance, in a wide range of mammalian tissues in addition to the endometrium; there it is concentrated predominantly in luminal and glandular epithelial cells of the uterine endometrium. Sin1 provides a possible link between type I IFN action, stress-activated signalling pathways, and control of prostaglandin production [79].

All these effects have motivated the search for human analogs. The rates of the secretion of interferon-α [80] are much too low, and their effects too classical, for it to be a suitable candidate. At one stage it was thought that the group of Imakawa [81] had identified an equivalent, named o-interferon, in the human [81], but this has never been confirmed. Recently, Zidovec et al. [82] have identified a putative candidate in form of an interferon-α-like activity in the human reproductive tract as well as early embryonic stages.

The Uterine Side

In the context of the Medawar 'tolerance to allograft' paradigm [83], but also on eve of the final enunciation of the Th1/Th2 proposal [84], most first saw with a certain surprise at the beginning of the 1990s that, whereas the implantation uterus was viewed by most immunologists as one of 'tolerance of the fetal allograft', with an absence of classical lymphocyte cell trafficking (hence the immunorepulsion theory), the postcoitum uterus was the transient siege of an intense cellular influx and inflammation-like reaction. (Hence, we organized a symposium in 1994 [see proceedings in 85].)

What was indeed seen first is that immediately after a successful mating, the uterine horns in rodents[1] are filled with T cells, B lymphocytes, and macrophages. Such trafficking is dependent upon the presence of an active ejaculate, since mating with a vasectomized animal does not elicit such a reaction. The pattern observed is very close indeed to what is seen during the inflammatory stage of an abscess after injection of a foreign substance [86, 87].

This should not really have been a surprise, since mating results in the intravaginal and cervical injection of millions to billions of foreign cells, not only MHC class I- and II-negative spermatozoa (but those express their antigens, especially S, P, and T which are the main targets in case of female 'auto'-immune antibodies in antibody-mediated female sterilities), but also millions of residing MHC+ (class I and class II) resident lymphocytes and macrophages. Only one of these cells will survive, in a distorted fashion by this kind of fusion which is the fertilization of the oocyte. All others die on site, hence the need for a cleaning of the site done by macrophages, activated in that process, which are the main cells migrating massively towards the uterine lumen.

As activated macrophages, they secrete by days 1–3 a series of inflammatory cytokines, mostly IL-1 and CSFs. Blocking of such an inflammatory reaction in one uterine horn prevents successful implantation in this horn, whereas the contralateral one remains receptive [86]. The cells, once their cleaning task is performed, retrocede away from the uterus, so that there does not remain much by day 2.5/3.

But studies of the implantation uterus itself have revealed, even more surprisingly for the time, that at that time the production of inflammatory cytokines continues to rise in the uterus, meaning that they are produced by nonimmune cells, e.g. within the stroma, which further immunohistochemistry has confirmed. Amongst these, IL-1, IL-6, CSF-1, GM-CSF, G-CSF could be noted, and even more surprisingly TNF and γ-interferon, both cytokines which

[1]Very obvious ethical constraints as well as technical ones render such a study impossible in humans, and also in the very expensive primate models.

are abortifacient in established pregnancy [87, 88, 89]. Here again, this reaction was not seen in the case of a mating with a vasectomized male [89].

Simultaneously, the Pollard group [5–7, 90, 91] demonstrated the role of CSF-1 in the uterus and the presence, in murine species, of c/fms, the CSF-1 receptor and oncogene, precisely in the MHC+ spongiotrophoblast area of the placenta. Indeed, this was followed by studies confirming the important role of progesterone in inducing CSFs in the uterus [92, 93], important growth factors for the trophoblasts, and the demonstration by Tartakowski et al. [94, 95] that CSF-1 could rescue embryos from early implantation failure while being toxic at high doses. This has prompted the search by neutralization and/or KO of the research of the role of these inflammatory cytokines.

IL-1

Simon et al. [96] initially claimed to have obtained a total block of implantation by acting at the IL-1 receptor level by injecting IL-1 receptor antagonists. This has never been reproduced in its original form. There is an ongoing controversy since further papers by Simon et al. retract the 100% effect in his colony in Spain, but maintain that he still obtains a high rate of implantation failure, while for example C. Stewart has not observed any implantation defects and reproductive failure in a variety of strains of IL-1 KO at Roche (pers. commun. and presentation at several meetings).

There are a variety of explanations for the variations observed, one being the presence of a trace of LPS in the contamination, which could induce enough TNF to be abortifacient as in experimental systems [97, 98], and the others being environmental differences (CBA/J × DBA/2, the classical murine abortion system [99], is very environment sensitive, for example [100]).

Much less controversial, Simon and his group [101–105] have convincingly described an IL-1 system in humans, whose distribution (receptor and interleukin) on uterine and placental tissues is indeed very suggestive of autocrine and/or paracrine effects as suggested as early as 1990 by Kauma et al. [105] or Frank et al. [106].

Amongst the (many) effects of IL-1, let us mention that IL-1 seems to influence (as IL-6) hCG secretion by the very early embryo as it does indeed for first trimester trophoblasts [107].

LIF

LIF was the *first* cytokine that was shown to be absolutely necessary for implantation [108–110]. Prompted by the discovery that LIF peak in the murine endometrium after progesterone administration was coincidental with the murine implantation windows, Stewart et al. [109] proceeded to their now classical experiments showing that LIF KO results in total implantation failure, and

that even LIF+/LIF+ embryos (which do secrete LIF at early stages) do not implant in a KO LIF–/LIF– mother.

LIF–/LIF– embryos, after flushing from a LIF–/LIF– mother, and transfer, do implant in LIF+/LIF+ or even LIF+/LIF– mothers. This defect was indeed highly suggestive of the key role of maternal LIF and it was proven by correction of the defect via an osmotic pump administering recombinant LIF [110]. These data have been confirmed independently; Conquet et al. [111] designed LIF KOs for developmental studies, which to their surprise showed the same reproductive defects.

The coincidence of the peaks of LIF in human endometrium with the timely appearance of the LIF receptor on the human blastocyst [112, 113] prompted the search for defects in humans. An association of LIF deficiency and sterility was indeed traced, by measurement of LIF production in the supernatants of endometrial explants [114, 115]; the data was later confirmed by flushing studies of Laird et al. [116].

The quantitative defects could eventually be linked to a local regulation, which makes sense since otherwise the LIF-deficient women would harbor several developmental problems as indeed noted in mice by Conquet et al. [111]. The dysregulation could be abnormal progesterone responsiveness, or, as was traced in vitro, a lack of CSF-1 or, in line with Simon's observations, deficient local IL-1 or deficient responses to these cytokines, which were shown to be LIF inducers in a progesterone-prepared environment [115].

Indeed, except for a study by Hambartsoumian [117] (who 'interpreted' data generated by Delage and Moreau[2]) LIF production in humans is boosted by progesterone in humans [118] as well as in animals. The important difference is that in mice it is produced in the uterus only during the preparation of the uterus for implantation, whereas in humans, while indeed peaking in the second half of the cycle and at the implantation window, it can be traced throughout the cycle and indeed throughout pregnancy.

Qualitative defects have been spotted, too: Giess et al. [119] found putatively inactive LIF mutations in some sterile women, and similarly Steck et al. [120] recently observed similar cases. The presence of such mutations could explain the presence of LIF 'high producers' in the sterile population [114], but of course the obvious alternative explanation for the latter phenomenon is that not all sterilities are due to LIF deficiency.

Another explanation could rely on which isoform of LIF is dosed by the commercially available ELISAs, and differences of sensitivity in the commercial

[2]This resulted in the refusal to co-sign the paper by both myself and J.F. Moreau who had done all the ELISAs and us sending a letter to the editor of the *American Journal of Reproductive Immunology*.

kits [121]. Indeed, LIF organization of the gene is complex [122] and the use of human cell lines has confirmed the existence of three isoforms, only one of which is active or displays the highest activity in the reproductive tract [123]. Only the soluble isoform seems in our hands to be necessary for successful implantation and thus the large scale trial of recombinant LIF which was recently performed in sterile women by a pharmaceutic company was doomed to fail because IHC labelling of LIF on tissue sections, for example, and the absence of proper selection (LIF sterilities are likely to represent a small fraction of female infertilities) were not setting up the proper conditions for observing any significant differences.

In our hands, too, excess LIF and an abnormal localization seem to impeach human implantation [124]. There are several possible explanations for this: whereas stromal LIF and likely moderate amounts of luminal LIF are useful, excess stromal LIF might lead to blocking the LIF receptors on blastocyst, preventing a blastocyst/membrane-bound LIF interaction, or it might simply reflect that, indeed, we correlate high levels of LIF with too high a number of NK cells and TNF. Thus, as for excess levels of CSF-1, which are abortifacient, such excessively high levels of LIF might reflect (or trigger) an ongoing 'chronic Th1 response', whereas the normal course of events required for successful pregnancy is the relapse of the pre- and peri-implantation inflammation so as to establish a 'tolerance-prone uterus', this is characterized by a high placental IL-10 secretion (placenta is the main source of IL-10 in the pregnant womb [126]), though NK cells and γδ T cells have also predominant IL-10 secretion during pregnancy [127]. Within the placenta, the source of IL-10 is the placental trophoblast [128].

The mechanism by which maternal LIF exerts its effects are nor really known. From the need of expression of LIF receptor on embryos [109, 112, 113], one might deduce that it acts mostly on the embryo proper. Indeed, except one study [129], all reports describe positive effects of LIF when added to embryo culture medium on blastocyst formation and embryonic appearance, be it in murine, bovine, ovine, or human species [130–137], with even in the murine species an enhancement of implantation rates and offspring [128] but objective molecular criteria are lacking.

LIF is expressed by the embryo, but also in the endometrium [109, 111, 113, 139]; an action there is indeed possible, as suggested by the enhancement of the survival of human endometrial cells in culture [140].

It could finally act on adhesion molecule expression and regulate integrin chain balance, which are important determinants of implantation success [10, 141–145] as many other cytokines described here might indeed do. Whatever the mechanism, exploration of LIF by flushing [116, 146] and/or microbiopsy can be performed as late as during the egg retrieval period without affecting future pregnancy rates [148]. Such an exploration should therefore – with the appropriate ELISA – be performed in sterile women.

IL-11

Another very important cytokine demonstrated by KO studies in mice is IL-11. Indeed, the spatial and temporal distribution of IL-11 in mice is already very suggestive, especially its appearance in the early stroma [147] but key studies were obtained by KO mice for the IL-11 Rα receptor, showing impaired decidualization as a cause for infertility in these animals [148–150].

CSFs

The role of CSF-1 and M-CSF has already been dealt with above [5–7, 90–96], but key molecules are also two of the first 'immunotrophic molecules' [2–4], which were originally the first recombinant molecules shown as active in a murine model of early fetal loss [4]. GM-CSF is not only secreted by T cells as well as uNK cells, but it is also, as CSF-1, present within the stroma [151, 152] where it acts as a growth factor but also prevents apoptosis of embryonic cells [153].

More important perhaps even, in view of the discussions on preeclampsia [154–158], and especially in the light of the effects of sexual cohabitation with a specific male partner [156–158], Robertson et al. [159, 160] have demonstrated a role for semen in the induction of early maternal antipaternal hyporesponsiveness, implying both GM-CSF and TGF-β as tolerogenic immunodeviating signals/molecules in the seminal fluid [160–164]; the seminal fluid is also involved in GM-CSF local production [162] and the MHC specificity is likely brought by paternal peptides coming from the afore-mentioned macrophage 'cleaning' of the postcoitum uterus [86, 87], and thus 'peptide loading'.

GM-CSF, as indeed IL-3, is also secreted in the early preimplantation and peri-implantation metrial gland [163] as well as by CD56+bright uNK cells in humans [166], and in the human and rodent uterine stroma [126, 165–167]. A classic 'immunotrophic molecule' [2–4] has been shown to have protective effects in the classical CBA × DBA/2 model [4], but also in the lupus Ac antibody-mediated fetal loss [168]. Whether this is due solely to immunotrophism or to it belonging to the classical Th2 cytokines [169] remains to be established; it is like other Th2 cytokines quantitatively deficient in the CBA × DBA/2 combination [64].

IL-6

Not only has embryonic IL-6 been implied in implantation, but IL-6 is an important molecule secreted by the peri-implantation uterus. Besides the role that some believe it plays in the generation of asymmetric antibodies it has been shown to regulate hCG production by early trophoblasts and trophectoderm and this in conjunction with its soluble receptor [170–175].

This pathway can be downregulated by TGF-β1 [176] and in a feedback fashion by hCG itself [177]. It is also known to regulate placental lactogen secretion (hPL) [178] and both hCG and hPL are known to inhibit IL-2 secretion and act as inducers of TNF [179]. Thus, they both act in an autocrine loop since TNF itself is known to act on hCG via IL-6 secretion [180], a further caveat against the use of Remicade in implantation defects!

IL-6 acts on the production of matrix metalloproteases by the invading trophoblasts, thus playing a key role in the invasion process [181]. It is also involved in the regulation of prolactin secretion [182], which needs to be stressed here, since most of the prolactin secretion defects result in sterility by complex pathways involving excess TNF [183].

IL-6 can also be produced by locally allostimulated lymphocytes (possibly in the vicinity), thus adding itself to the 'immunotrophic loop' [184]. Its production is also upregulated by leptin [185], putting again emphasis on the newly discovered role of this molecule in implantation.

As for CSF-1 and LIF, excess IL-6 is *not* beneficial if present before fecundation: as an example see endometriosis, but effects are then on the follicular environment and embryo quality [186].

IL-4

IL-4 is one of the 'classical' Th2 cytokine. It is secreted mostly by the endometrium in the reproductive tract in humans [187–190] but it is also produced by transiting a Th as well as local NK cells [191] IL-4 receptors are detected in the placenta, where one can also observe its secretion, which is mostly if not exclusively coming from trophoblasts [189]. This latter scheme suggests an autocrine role. It is also secreted mostly in the uterus in mice [64, 147, 169]. There is indeed a deficient production of IL-4 in murine models of spontaneous abortion [64] as well as in cases of early pregnancy loss by decidua T cell clones [191–193]. In keeping with such functions, IL-4 regulates prostaglandin production in the uterus [194, 195], and interestingly also IL-1 receptor antagonist, which it downregulates [196], in keeping with the role postulated for IL-1 receptor antagonist by Simon et al. [96], but also MIP-1α.

As an example of variations between species, equine trophoblasts do not produce IL-4 [197].

Th3 Cytokines: TGF-β

TGFs, especially TGF-β1, are all present within the uterus [105, 198], especially at the implantation site(s) in the pre- and peri-implantation period [198]; they are also secreted by Th3 and γδ T cells [125, 199, 200] together with natural suppressor cell-like cells [199, 200] which produce mostly a

TGF-β2-related molecule [201] upon which the success of the immediate postimplantation period may depend [202–204]. As stated above, TGF-β present in the sperm has important immunodeviating capacities during the early implantation steps [162]. The signal transducers of TGF in endometrium peak during implantation [205], but are also present in the embryo where they regulate development [206].

The Main Th2 Cytokine: IL-10

IL-10 levels correlate with the success of pregnancy [64, 193, 207, 209] and an IL-10 injection corrects resorptions in the CBA × DBA/2 model [64]. It is secreted by a variety of cells in the decidua in the peri-implantation period [125–127, 164, 175, 191, 193], its main source being the developing placenta [127, 128, 130, 211], or in early pregnancy and in case of alloimunization NK cells [210, 211]. Indeed, it is especially important in the 'resorption window' after implantation. Even then, data quoted not too often but present in the original paper on its effects in the CBA × DBA/2 mating [64] showed that its neutralization in a *non*-abortion-prone murine mating combination did *not* affect the resorption rates. This was also known at the time of the IUIS/ICI Delhi meeting, when no effect of an IL-10 KO was seen in infertility, a fact confirmed in single and multiple Th2 cytokine KO mice [211–213]. It is interesting to note that the last paper concerns a KO mutation affecting four Th2 cytokines [213], albeit the authors obtained a Th2-like response with IL-4 alone and the KO did not affect IL-3, a Th2 cytokine promoting fetal survival [4, 168]. These data are amongst those that challenge the Th1/Th2 paradigm [214].

IL-13

The Th2-like cytokine IL-13 is present like a 'Th2' shield all around the peri-implantation embryo [124], but its KO does not affect fertility [213].

TNF

In the same vein, whereas there are ample data to show that indeed high doses of TNF are abortifacient be they LPS dependent, alone or in conjunction with γ-interferon [4, 64, 94, 98, 215–220], or as a consequence of stress [215] and that there is more TNF in abortion-prone matings early peri-implantation decidua than controls (and more γ-interferon) [216–219], there are also data showing that it plays other roles [199, see 220], including possibly as a protection against developmental toxicants [221].

These data, and the new role ascribed to γ-interferon and NKs, put importance on 'new' cytokines and vascular events [147, 214] as first seen when we were looking at IL-18 production in murine abortion models [222], and because of the renewed role of NKs.

New Cytokines: Vascularization and New Role for NKs

Implantation requires not only adhesion, but a proper decidual transformation so as to afford the nutritive elements essential for the embryo, but also neoangiogenesis so as to give it a proper vascular bed. The uterine arteries thus have to evolve to the spiral ones, and indeed it has been shown that many immune abortions, be they mediated by autoantibodies (lupus) or by TNF + interferon-γ, activation of the fgL2 prothrombinase pathway [98] are due to intravascular coagulation on the maternal side.

Indeed, angiogenic factors are essential in this process, and the lack of VEGF blocks implantation [223].

In this context, the Th1/Th2 paradigm, established for an ongoing pregnancy, postulated that Th1 cytokines were abortifacient, Th2 protective, and that NKs, as effector cells in abortion, should be downregulated [64, 84, 207–210]. Indeed, studies had demonstrated a role for activated NKs in immune abortion [216, 224–228]. In this context interferon-γ was seen only as a 'bad guy' mediating for some 'immunodystrophism' and thus implantation failure [229–232].

It is in this context that, to our surprise, it was discovered that 'new' (at that time) cytokines were expressed in the reproductive tract, their localization as well as their stage-specific expression being highly suggestive of an important role in implantation [147].

In the meantime, Croy and colleagues [233–235] had demonstrated that NK KO mice displayed severe problems of growth retardation, and that this was linked to abnormal vessel development. Proof that NKs are involved came from the reconstitution of NK-depleted mice, which corrected their reproductive performance [235]. It must be mentioned here that IL-2 common receptor γ chain KO mice studied by Saito and his group [236] have a normal pregnancy. Similarly, IL-15 is a growth factor for uterine NK cells in mice and humans, which also activates the cells to possess a high lytic potential, notably granzyme and perforin [237–241], but in IL-15 KO mice there is no fetal death, and they are surprisingly resistant to Listeria; however, in agreement with the studies by Croy et al., they display abnormal uterine arteries [242]. The reasons for such discrepancies as regards fetal viability are likely that we do not fully understand the uNK subsets, themselves very different from peripheral ones [243].

These data were taken by us as an explanation for the distribution of IL-12 and IL-18 in the murine uterus at the pre- and peri-implantation period [147, 222] with more IL-18 in *non*-abortion-prone matings than in abortion-prone ones [222]. Indeed, IL-12 can activate NKs, and, especially in conjunction with IL-18, lead to cytotoxic events and abortion, but it is also a factor acting on angiogenesis [244].

In fact, monocytes are primed to produce IL-12 in normal pregnancy [225]. As for CSF-1 and LIF, there are dose effects: there is too much IL-12 in preeclampsia, and IL-12 seems downregulated in abortion [246] (which is incompatible with the 'explanations' put forward by practitioners of immunotherapy by lymphocyte alloimmunization), while there is too much IL-12 in preeclampsia and high levels of IL-18, IL-12, or IL-18 + IL-12 are abortifacient or induce preeclampsia [247–250].

The requirement for NK activation in achieving proper vascular flow stems from the fact that besides VEGF, another cytokine is required for proper local angiogenesis, angiopoietin 2, and this is produced by (IL-12, IL-18) activated uterine NK cells [251, 252].

It is in this context that γ-interferon is required for murine uNK activation [253, 254], at low doses at the time of implantation (which are precisely observed- as a consequence of the aforementioned postcoitum events) [86–89]. In such a conception, an optimal IL-12 + IL-18 level is required for successful implantation.

It is precisely what we find in humans [255]: either a lack of IL-18 production and/or IL-12 secretion, or conversely levels of these which are too high, and abnormal localization correlate with abnormal vascular scores, likely linked to an absence of proper transformation of uterine spiral arteries.

In fact, data obtained when studying IL-12, IL-18 and interferon-activities, in keeping with the concept that the Th1/Th2 paradigm is an oversimplification, and as suggested in earlier studies in mice [173], show that not only quantitative defects, but also changes in the distribution pattern will determine whether a cytokine would exert positive or negative effects [253] reminiscent of the aforementioned observations in the LIF system [124].

Further studies have been conducted exploring the complete IL-12/IL-15/IL-18 system, which requires quantification of the two isoforms of IL-12, the homo- and heterodimers, whose frequency can be estimated by quantifying the p35 and p40 chains, IL-15, IL-18, IL-18 binding protein (IL-18 BP) and EBI3, an IL-12 antagonist [256]. IL-15 had to be included, because it is widely expressed at the murine interface [147], and also in the metrial gland there [242], where it regulates granzyme and perforin content [240], and because at high doses and in the p70 form, it activates uNKs to become lytic for trophoblast-derived cells [257]. IL-18 BP was known to be expressed in human endometrium [258], but its functional significance had not been probed.

The results [259] correlate IL-15 levels with high IL-18 levels in sterile women, but not with excess NK counts, so that the main effect of IL-15 should be more on NK activation [240], rather than replication, not excluding an effect on non-uNK cells. The number of uterine CD56 bright cells was rather strongly correlated to the ratio of IL-18/IL-18 BP.

It is worth recalling in this context that in the Croy studies [260, 261], as well as in the recent ones from Barber and Pollard [242], IL-15 is indeed required for uNK cells, but a T cell component, and likely 'activation' of NK by IL-15, is indeed required to correct NK deficiency-associated placental growth and vascular defects. It should also be mentioned that IL-15 might regulate per se trophoblast invasiveness [262].

These studies put the emphasis on control of the NK activation, and the early 'choice' between a lytic and/or trophic/angiogenic pathway. It is important to mention in this respect that besides VEGF and angiopoietin 2, 'old' molecules such as hCG (themselves partly regulated by IL-6 and other cytokines; see above) have indeed recently been reported to be angiogenic [263].

Conclusion

At the end of this review, where it should be recalled that we have investigated neither the complement deficiencies nor IDO dysregulation [264, 265], we believe that new ways are opening for implantation studies: investigating the role of cytokines at the inflammatory stage required for apposition and adhesion events and the subsequent regulation of adhesion molecules [266], continued by the simultaneous expression – under cytokine regulation – of enzymes controlling invasiveness, defining the relation between well-known cytokines and emerging centers of interest such as leptin [267], delineating the minimal infectious signal (called by some 'danger signal') for abortion [268] are obvious objects of interest. But, above all, coupling PCR evaluation of cytokines [259], flushings [116, 124] and screening or embryo supernatants (see above) for cytokine content with an estimation of the development of the uterus and its vascular flow [269–277] should make it possible to arrive at the 'biochemical diagnosis' of pregnancy as well as possibly 'biochemical embryonic preimplantational screening' [278].

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T Helper 1 and 2 Immune Responses in Relationship to Pregnancy, Nonpregnancy, Recurrent Spontaneous Abortions and Infertility of Repeated Implantation Failures

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Abstract

It is becoming clear that during each developmental stage of pregnancy, different immunological conditions exist and may even be necessary for success. The widely accepted T helper (Th) 1 and 2 concept has some limitations if applied to the various developmental stages of pregnancy. During the implantation period, a multidirectional cytokine network is necessary with the blastocyst producing cytokines and other factors and the endometrium synthesizing factors necessary for the embryonic development. Improper immune responses and an unbalanced cytokine network may be related to implantation failures, pregnancy losses and obstetrical complications. A propensity to Th1 immune responses has been reported in these conditions systemically or locally. The presence of elevated Th1:Th2 cell ratios, high concentration of Th1 cytokines secreted by peripheral blood mononuclear cells, elevated NK cell cytotoxicity and levels, and emergence of various autoantibodies are the supporting evidence. The underlying immunopathology for the preponderance of Th1 is unknown. Genetic, environmental, and hormonal etiologies need to be explored further in the future. The purpose of this review is to give an overview of what is known about the immune response in women with reproductive failures and provide an update of some of the most recent findings in this field.

Introduction

CD4+ T helper (Th) cells can be classified into different types based on their cytokine profile. Cells with these polarized patterns of cytokine production

have been termed Th1 and Th2. Th1 cells produce IL-2, interferon-γ (IFN-γ) and lymphotoxin (TNF-α) and promote delayed-type hypersensitivity responses. Th2 cells produce other cytokines including IL-4, IL-5, IL-10 and IL-13, which direct allergic or anti-inflammatory responses as well as helping B cell responses [1]. The generation of Th2-type cytokines has a pivotal role in allograft tolerance, whereas cellular immunity plays a crucial role in acute allograft rejection [2]. Embryos normally are not rejected by the maternal immune system despite the presence of paternal major histocompatibility complex antigens [3]. This can be partially explained by a Th2 bias which protects the embryo against the maternal rejection. Although the role of Th1 and Th2 cytokines in pregnancy has been well characterized as described below, other cytokines such as IL-12, IL-17, IL-18 and leukemia inhibitory factor (LIF) have been identified and their role in pregnancy success or failure is also being evaluated [4–7].

It has been demonstrated in animal models that normal pregnancy requires a propensity to Th2 immune responses [8–10]. In the mouse, recurrent abortions in a DBA/2 × CBA/J mating are mediated by TNF-α release in the decidua and abortions can be hindered by the induction of Th2 cyokines such as IL-10 and Th3 cytokines, and transforming growth factor-β2 (TFG-β2) which is secreted by γ/δ T cells. Natural killer (NK)-γ/δ T cells may be quite important in the Th1 response in early pregnancy that predisposes to abortions in CBA × DBA/2 mating, whereas γ/δT-only cells appear to be protective. An activating stimulus (such as stress or endotoxin) appears to be as important in triggering abortions, as is the Th1/Th2,3 ratio at the fetomaternal interface [11].

Recent data from the human experience suggest that Th1 immune responses are detrimental for pregnancies and Th2 immune responses are necessary for successful pregnancies. Significantly higher serum levels of Th2 cytokines IL-6 and IL-10 were detected in normal pregnancy compared with unexplained recurrent spontaneous abortions (RSA) and significantly higher serum levels of the Th1 cytokine IFN-γ were present in women with RSA compared with normal pregnancy [12]. Lately, flow-cytometric single cell analysis of cytokine production represents a powerful approach to investigate such effects since it permits the simultaneous detection of surface molecules (immunophenotyping) and intracellular cytokines in individual cells and thus allows the analysis of the heterogeneous populations of cell subsets that can lead to variations in cytokine profiles [13]. This technical advance allows the precise investigation of Th cells and their subpopulations in human reproduction. Infertility, repeated pregnancy losses and certain obstetrical complications have been reported to be a consequence of the preponderance of Th1 cytokines [14, 15]. Women with RSA and infertility of multiple implantation failures demonstrated significantly elevated peripheral blood Th1/Th2 cell ratios as compared to those of normal fertile controls [14]. Patients with preeclampsia

have significantly higher expression of IL-2 in peripheral blood mononuclear cells (PBMCs) when compared with that of women with uncomplicated pregnancy. Furthermore, in the group of patients with preeclampsia the expression of IL-2 were higher in CD8+ T lymphocytes than in CD4+ T cells. The expression of IL-10 was lower in lymphocytes of preeclamptic patients when compared with controls [15].

Although a Th1/Th2 imbalance is often suggested to be a fundamental paradigm for specific disease-related immune responses, when examined closely, many of its features become untenable [16]. In addition, a rigorous evaluation of the types of Th1 and Th2 cytokines expressed in patients with fetuses of ill-defined gestational ages in previous studies led to the incorrect application of the Th1/Th2 paradigm at various stages of pregnancy without careful consideration [17]. For an assessment of pregnancy-related immune responses, we suggest that the following facts should be considered: (1) the nature of Th1 and Th2 cytokines is varied, (2) systemic and local immune responses (endometrium) may not always be synchronized, (3) each developmental stage of pregnancy may need a different immune response, (4) immune responses are often reciprocal between mother and fetus, and (5) measuring the immune response using absolute versus relative levels may result in different conclusions.

In this review, the normal immune responses that occur during the menstrual cycle, and during the peri- and postimplantation period are discussed followed by a review of the local and systemic immune responses found in women with RSA and infertility of implantation failures.

Menstrual Cycle and Immune Responses

Immune variation during the menstrual cycle has been attributed to fluctuations of female hormones [18]. Differential susceptibility to autoimmunity between the sexes, changes in autoimmune disease activity during the menstrual cycle and in pregnancy, and in vitro studies of hormonal influence on cytokine production are a few examples of immune differences mediated by female hormones [19]. One study even suggests that an autonomic balance shifts towards a sympathetic bias during the second half of the menstrual cycle with regard to cardiac function [20]. This shift can be seen as an evolutionary adaptation to address the immunological and physiological demands for successful implantation and gestation. It has been suggested that through direct modulation of the lymphoid system and activation of the cortisol pathway, a sympathetic bias promotes a shift to relative Th2-biased immunity which may favor maternal tolerance of the embryo by attenuating the Th1-mediated interference of implantation [21].

In the menstrual cycle, a difference in the expression of immune-related genes during the follicular and luteal phases was observed in peripheral blood lymphocytes, which reflects a difference in a systemic response. The Th1 immune response was significantly downregulated in the luteal phase, which was accompanied by downregulation of a significant number of IFN-related genes [22]. During the perimenstrual (3 days prior to 4 days after onset of menses) interval, PBMC produce less IFN-γ and more IL-10, resulting in a decreased IFN-γ:IL-10 ratio compared with the mid-cycle interval (days 13–16). This change was not seen in women taking the oral contraceptive pill, which suggested the role of ovarian steroids in cytokine modulation [23]. Humoral immunity also changes during the menstrual cycle. The immunoglobulin secreting cell frequency in PBMCs (another systemic response) is highest during the periovulatory stage of the menstrual cycle [24]. These results support a significant hormonal regulation of the immune system and therapeutic implications in infertility or autoimmune disease in women.

The regularly menstruating endometrium expresses various cytokines and chemokines, such as IL-1, IL-4, IL-5, IL-8, IL-10, IL-13, IL-15, TNF-α, IFN-γ, monocyte chemotactic protein-1 (MCP-1) and regulated upon activation normal T cell-expressed and -secreted (RANTES) protein and perhaps many more [25, 26]. Withdrawal of progesterone from an estrogen-progesterone-primed endometrium is the initiating event for the cascade of molecular and cellular interactions that result in menstruation. Progesterone withdrawal first affects cells with progesterone receptors. Early events in the menstrual process are vasoconstriction and cytokine upregulation [27]. Later, progesterone withdrawal results in a local increase of inflammatory mediators and the enzymes responsible for tissue breakdown [18]. As evidenced by intracellular staining, IFN-γ is produced by both stromal cells, possibly polymorphonuclear neutrophils, and intraepithelial lymphocytes through all stages of the menstrual cycle. It is noteworthy that polymorphonuclear neutrophils produce IFN-γ in response to the stimulation of TNF-α, IL-12 and LPS [28]. IL-13 and IL-15 mRNA and protein are expressed in endometrium throughout the menstrual cycle with peak expression detected immediately after and prior to onset of menses. The ratio of IL-13:IL-15 expression revealed a predominance in IL-13 expression during late proliferative/early secretory phase and IL-15 during the midsecretory phase [29]. Thus a differential expression of cytokines can be observed in the endometrium during the menstrual cycle.

Cytokines in the endometrium also affect the tissue-remodeling process by acting on the matrix metalloproteinase enzyme (MMPs) promoter, either independently or in consort with steroid hormones, to provide both positive and negative regulation of these genes [30]. Menstrual fluid contains precipitable rheumatoid factor, as well as immunoglobulins IgG, IgM, IgA and IgE,

predominance of IgG and rheumatoid factor. A possible role for a physiological mechanism to remove the unnecessary antibodies formed due to fetal allograft or intrauterine infection has been proposed [31].

Peri- and Postimplantation Endometrium

The endometrium goes through sequential changes during the menstrual cycle and pregnancy, which include not only histological changes in stromal and glandular cells but in leukocyte trafficking. Factors in seminal plasma may play a role during early pregnancy and in regulating preimplantation embryo development. In the pig model, these factors elicit an endometrial inflammatory infiltrate comprised of predominantly activated macrophages and dendritic cells. Seminal plasma induces the expression of three cytokines, granulocyte macrophage colony-stimulating factor, IL-6 and monocyte chemoattractant protein-1, and the eicosanoid-synthesizing enzyme cyclooxygenase-2 [32]. Human seminal plasma contains TGF-β1, IL-8 and vascular endothelial growth factor (VEGF), >10-fold above the serum level [33]. Incubation of endometrial epithelial cells with seminal plasma resulted in concentration-dependent stimulation of IL-1β, IL-6 and LIF mRNA expression. Maximum stimulation was found in midsecretory phase epithelial cells. This effect might at least in part be exerted by TGF-β1 and IL-8, abundantly present in seminal plasma and also affected by endometrial dating [34].

Prior to the implantation, an embryo sends the signal to the endometrium. In situ hybridization revealed IL-1α and IL-6 mRNA localization in normal, dormant and activated blastocysts and a differential expression was observed in relation to the exposure to progesterone and estrogen in mouse blastocysts during implantation [35]. The expression levels of LIF and LIFR in uterine epithelium are gradually increased during the preimplantation period and reached their highest levels on day 6.5 of pregnancy, just before the time of blastocyst implantation in the rabbit model [36]. These findings suggest that a paracrine LIF or cytokine circuit should exist between the endometrium and the early embryos for successful implantation.

Apoptosis is critical for the appropriate tissue remodeling of the maternal decidua and invasion of the developing embryo, yet the regulation of apoptosis is also imperative for a successful pregnancy. The quick and effective removal of apoptotic cells by tissue macrophages represents an essential process, which prevents the release of self-antigens, and in the case of pregnancy, paternal alloantigens. Recent studies have shown that the process of apoptotic cell clearance is not a neutral event, but rather an active one that induces macrophage production of anti-inflammatory cytokines and survival factors [37]. Apoptotic cell clearance is,

therefore, necessary for the resolution of inflammatory conditions, which during pregnancy could have lethal consequences.

After the implantation hCG seems to play a major role in immune regulation. hCG administration during the secretory phase significantly modulated several endometrial paracrine parameters that correlate to endometrial differentiation (IGFBP-1), angiogenesis (VEGF), implantation (LIF, M-CSF) and tissue remodeling (MMP-9) [38]. The ubiquitin-proteasome pathway also plays an important role in embryo implantation. LMP2 and LMP7 (LMP = low molecular mass polypeptide) are the two subunits of 20S proteasome, which are critical for proteasome activity. Possible roles in trophoblast invasion, angiogenesis, degradation of extracellular matrix and immune tolerance were suggested in a rhesus monkey model [39]. After the implantation, cytotrophoblasts can attract monocytes and $CD56^{bright}$ NK cells by producing MIP-1α, and it is hypothesized that these cells may organize and act on leukocytes at the maternal-fetal interface [40]. Recently three cytokines, IL-16, IL-17, and IL-18, were detected at the mouse fetomaternal interface with a tissue-specific, stage-dependent distribution. The predominance of IL-18 secretion in the nonresorption-prone matings raises the question of the general validity of the classical Th1/2 paradigm [6]. It would be interesting to determine whether there was a correlation with the expression of these cytokines and pregnancy loss or infertility. Determination of these three cytokines with regard to systemic or peripheral expression is apparently not known.

Cytokine Regulation in Women with Infertility

Following the determination of systemic or peripheral Th1/Th2 cytokine cell ratios, women with infertility with multiple implantation failures after IVF cycles demonstrated elevated Th1/Th2 cytokine-producing cell ratios [14, 41]. This imbalance was evident when the Th1/Th2 cell ratios were compared between the women with multiple implantation failures after IVF/ET and normal fertile controls. The proportion of TNF-α-producing CD3+/CD8− cells ($p < 0.05$), and the Th1/Th2 ratios of TNF-α/IL-4 ($p < 0.05$) and TNF-α/IL-10 ($p < 0.005$) in CD3+/CD8− cells were significantly higher in women with multiple IVF failures without spontaneous abortions as compared with those of fertile controls [14]. In addition, IL-10-producing CD3+/CD8+ T cell counts were significantly lower ($p < 0.05$), and TNF-α-producing CD3+/CD4+ T cell counts were higher in women with implantation failures ($p < 0.005$) than those of nonpregnant fertile controls [41]. These women also demonstrated significant alterations in NK cells and CD19+/5+ B cells. A significant increase in CD69 expression on CD56(+) NK cells was demonstrated in women with RSA

($p < 0.005$) and infertility ($p < 0.05$) as compared with that of normal controls. Conversely, CD94 expression was significantly decreased in women with RSA ($p < 0.005$) and infertility ($p < 0.05$) in comparison with that of controls [42]. The conception rate of women with normal CD56+ NK cell levels was much higher than that of women with elevated CD56+ NK cell levels [43].

The possible roles of genetic mutations, stress, ovulation stimulating agents, nonphysiological levels of ovarian steroids, or endometrial stress product were suggested to be causally associated with elevated Th1 immune responses in these women [14]. Stress due to longing for parenthood, depression and anxiety are common feature of these patients [44, 45]. It has been reported that stress increases lymphocytes, granulocytes, NK cell, NK cell cytotoxicity and decreases CD3+, CD4+ and CD19+ cells during the follicular phase, even in healthy women [46]. A potential Th1 induction by gonadotropin-releasing hormone (GnRH) has been reported. A marked increase in IFN-γ and inhibition of IL-4 production from lymphocytes of pregnant rats treated in vitro with different doses of GnRH-Ag has been reported. IL-2 and IL-10 production in response to GnRH-Ag were not different. This suggests an additional function for GnRH as a Th1 inducer and Th2 inhibitor. GnRH can thus skew the cytokine balance to predominantly Th1 type in pregnancy, leading to the termination of pregnancy in rats [47]. In women with a long GnRH analog protocol during the controlled ovarian hyperstimulation (COH) cycle, whole blood culture IL-2 levels were increased significantly during COH until peak E2, and then decreased significantly after hCG administration. IL-2 levels were decreased in the control and PHA culture media on day ovum pickup compared with day hCG [48]. It is plausible that a Th1 shift in women with infertility may be induced by repeated COH or that a preexisting Th1 shift can be even further aggravated while they are on COH with GnRH Ag. Preconception treatment of high-dose immunoglobulin G, which suppresses Th1 immune responses [49], has been reported to improve implantation rate and pregnancy outcome [50]. Further investigation may open new therapeutic approaches for women with Th1 shift and infertility of implantation failures.

Recent studies suggest LIF may also play an important role in human embryo implantation; LIF endometrial production is regulated by progesterone both in vivo and in vitro [51]. However, potentially functional mutations in the LIF gene do infrequently occur in women with unexplained infertility. Routine screening for LIF mutations or polymorphisms in these women may not be justified due to the low prevalence of gene alterations [52]. In women with IVF/ET cycles, LIF was detected in uterine flushings in 46% of patients at the time of egg retrieval, but no correlation were observed with better pregnancy rates in patients with detectable LIF. The mean LIF level did not differ in pregnant and nonpregnant women [7]. More recently uterine flushings were measured for IL-18 levels and a positive predictive value was found [53].

IL-12 is a dominant factor in driving the development of Th1 cells from antigen-specific naive CD4+ T cells and IL-18 has a role in inflammatory cytokines. Ledee-Bataille et al. [4] reported that the fertile women displayed normal endometrial vascular parameters, a weak anti-IL-12 staining, a consistent moderate stromal anti-IL-18 staining, and fewer than 15 NK cells/field. This pattern was observed among only 17% (6/35) of the women with implantation failures. Thirty-seven percent (13/35) of them had more than 40 NK cells/field with a strong anti-IL-12 and/or anti-IL-18 staining. Interestingly, 85% of these women displayed abnormal vascular parameters. The remaining 46% (16/35) had a marked local depletion of IL-18 and IL-12. Thus, evaluation of the immune responses in the local endometrial environment by biopsy or by uterine flushing may result in a higher success rate of IVF.

Pregnancy Losses and Cytokine Networks

While there is a relative paucity of data from human pregnancies indicating that Th1-type immune effectors actually lead to pregnancy loss, there is some compelling evidence linking inappropriate Th1-type immunity to pregnancy loss [8]. Significantly higher concentrations of Th2 cytokines (IL-4, IL-6 and IL-10) were produced after 24 h culture by PBMC from first trimester normal pregnant women than from women with RSA, while significantly higher concentrations of Th1 cytokines (IL-2, IFN-γ, TNF-α and TNF-β after 96 h PBMC culture) were produced by women with RSA as compared to first trimester normal pregnant women, indicating a distinct Th2 bias in normal pregnancy and a Th1 bias in unexplained RSA [54]. It has been demonstrated that the stimulation of maternal PBMC with autologous placental cells in vitro results in a Th1-biased production of cytokines in women undergoing unexplained RSA [12]. This is mirrored by the situation at the maternal-fetal interface shown by Piccinni et al. [55]. Significantly higher concentrations of IL-4 and IL-10 were produced by T cell clones from the decidua of women with normal pregnancy than from recurrent aborters. In addition, abortion-prone women who proceed to have a successful pregnancy are more Th2 biased than abortion-prone women who abort, and recurrent aborters who undergo spontaneous abortion have a stronger Th1 bias than aborters who have a normal pregnancy [56].

Flow-cytometric intracytoplasmic cytokine expression analysis has revealed detailed phenotypic information about Th1 and Th2 cells. We have reported that IL-10-producing CD3+/CD8+ T cell counts were significantly lower ($p < 0.05$) and TNF-α-producing CD3+/CD4+ T cell counts were higher in women with RSA ($p < 0.05$) than those of nonpregnant fertile controls [41]. RSA women demonstrated significantly higher Th1/Th2 ratios of IFN-γ/IL-4

($p < 0.01$), TNF-α/IL-4 and TNF-α/IL-10 ($p < 0.05$ each) in CD3+/CD8− Th cells than those of controls [14]. The maternal Th response appears to operate independently of hormonal factors in influencing the success or failure of human reproduction, as no correlation was evident between serum hormone levels and cytokine levels [57].

Recently, there has been an increased interest in newly identified cytokines such as IL-12, IL-15, IL-16, IL-17 and IL-18, decidual NK cells and angiogenesis in relation with recurrent pregnancy losses and implantation failures. IL-12 is known to be a Th1 inducer cytokine. Elevated serum levels of IL-12, IL-18 and IFN-γ have been reported in pregnant women with a history of RSA who miscarried the index pregnancy as compared to normal pregnant women and women with a history of RSA who delivered the index pregnancy [58]. In addition, it has been shown that first trimester IL-18 serum level distinguishes between successful and unsuccessful pregnancies [58]. Also, IL-18 in cervical mucus and amniotic fluid was higher in women with preterm labor than in those not in labor, at term. Elevated IL-18 in amniotic fluid was correlated with microbial invasion of the amniotic fluid [59]. IL-18, originally designated as IFN-γ-inducing factor, has been reported to synergize with IL-12 in the induction of Th1 cells. Interestingly IL-18 can also induce T cells to differentiate into Th2 cells, in the presence of TCR activation, either alone or together with IL-4 [60]. Therefore, it is suggested that the elevated levels of IL-12 found in those who miscarried in conjunction with IL-18 can cause a Th1 response to dominate in these women [60]. IL-18 may occupy a crucial role in promoting either Th1 or Th2 responses. The synergistic impact of these two cytokines may explain controversial experimental data of IL-12, which demonstrate the failure to increase the pregnancy absorption rate after intraperitoneal IL-12 injection [5]. In mice, the importance of both IL-12 and IL-18 in the induction of spiral artery modification and IFN-γ synthesis has been reported. Significantly more IL-18 was present in decidua or placenta from two non-abortion-prone combinations of mice, BALB/c × BALB/k (H2d × H2k), and CBA/J × BALB/c [6]. IL-12 and IL-18 have an important role in implantation. However, IL-18 can synergize with IL-12 in the induction of Th1 immune responses, resulting in pregnancy losses.

Both IL-15 mRNA and protein were found in a variety of cells but particularly in decidual macrophages. IL-15 induces a proliferative response in decidual NK cells and augments the cytolytic activity of decidual NK cells against K562. Interestingly, in contrast to IL-2, although activation with IL-15 resulted in some killing of JEG-3 choriocarcinoma cells, normal trophoblast cells remained resistant to lysis. IL-15 is a candidate cytokine responsible for NK cell proliferation in vivo in the progesterone-dominated secretory endometrium and early decidua [61]. The decidual abnormalities, including thickening of the

arterial walls with luminal narrowing and a hypocellular decidua basalis in the IL-15(−/−) mice, did not result in infertility as gestation times and litter sizes were comparable to those of wild-type mice [62]. Interestingly, the endometrium of women with RSA express elevated levels of IL-13 and IL-15, with the IL-13:IL-15 ratio favoring IL-13. Immunoreactive IL-13 and IL-15 were localized primarily in endometrial luminal epithelial cells with an increased intensity in glandular epithelial and stromal cells [29]. In women with RSA, increased CD57+ NK cell infiltration was noticed at the placental implantation site and this finding demonstrates the presence of enhanced Th1-type cytokine networks [63].

Although the biological significance of these cytokines in human endometrium and their elevated expression in RSA await investigation, these cytokines with distinct biological functions may regulate endometrial inflammatory/immune responses, tissue repair and receptivity for embryo implantation.

Influence of Th1 and Th2 Cytokine Gene Polymorphisms in Pregnancy Loss

In recent years, it has been shown that cytokine gene polymorphisms can influence the levels of cytokines. This finding has led to studies regarding possible pathophysiology in various diseases. Some studies have investigated an association between cytokine polymorphisms and RSA. The polymorphisms studied have been primarily polymorphisms in promoter regions for genes encoding TNF-α (−308G→A), IL-10 (1082 G→A), IL-6 (a174G→C) and IFN-γ (+874A→T). These polymorphisms influence the production of each cytokine and result in high, intermediate or low levels of each cytokine. Several studies have indicated the strongest association is found for the high production polymorphism of IFN-γ and IL-10 (which may be paradoxical) [64, 65]. There also appears to be a trend for such an association with the gene polymorphisms controlling high TNF-α production and recurrent pregnancy loss.

In 2003 Daher et al. [66] studied 48 women with recurrent pregnancy loss and 108 controls for the presence of these polymorphisms; they found that there was a statistically higher percentage of the T/T high IFN-γ genotype and a trend toward the G/G high IL-10 and A/G high TNF-α genotype in women with recurrent pregnancy loss. However, these women represented about 25% of the women with recurrent pregnancy loss suggesting that they may form a subset of patients. A meta-analysis was performed on similar studies published up to the 2003 (one of which concluded there was no association) and concluded that the data indicated a significant association between high cytokine polymorphisms

for IFN-γ [T/T: odds ratio (OR) = 1.92, p = 0.04] and IL-10 (G/G: OR = 1.75, p = 0.03) and a trend for association with high TNF-α production A/A and A/G (OR = 1.61; p = 0.18).

Several other studies have been reported since the previous studies and the meta-analysis. One by Prigoshin et al. [67] found no association between TNF-α, IL-6, IL-10 and TGF-β but a significant association between the high IFN-γ cytokine polymorphism and patients with recurrent pregnancy loss in Caucasian Argentine women (patients 65%, controls 35.8%, p = 0.01). Their study also found a statistical decrease in the frequency of IFN-γ A/A low genotype polymorphism between patients and the control group (20 vs. 41.5%, p = 0.04). These authors pointed out that the inheritance of certain cytokine gene polymorphisms is strongly associated with ethnicity; this suggestion means that studies around the world with women strictly stratified not only for clinical findings but also for ethnicity may be necessary to completely evaluate the relationship between the polymorphisms for high, intermediate and low level production of certain cytokines. Indeed, an investigation of TNF-α gene polymorphisms from an Austrian group found no association between the TNF-α gene polymorphisms (TNF-α –308 and –863) and recurrent pregnancy loss or serum TNF-α blood levels in Caucasian women [68]. In contrast a population of Japanese women carrying the –634G allele of the IL-6 promoter gene had a decreased risk of recurrent pregnancy loss; this finding is consistent with the Th1:Th2 paradigm regarding pregnancy loss [69]. Thus although these data may support the hypothesis that high Th1 cytokine production (IFN-γ and TNF-α) or low Th2 cytokine production (IL-10 and IL-6) may adversely affect a pregnancy, more studies are required to evaluate the importance of these polymorphisms in pregnancy outcome.

Conclusion

'Nothing in immunology is ever as simple as it first seems' as Dent [70] stated, and understanding the role that a cytokine/chemokine network plays in the endometrium and during pregnancy needs constant reevaluation. The current Th1 and Th2 concept needs to be updated to accommodate newly identified cytokines and their roles in successful pregnancy. With the recent advances in flow-cytometric techniques, the presence of a systemic Th1 propensity has been documented in women with recurrent pregnancy losses and infertility of implantation failures. Recent interest in IL-12, IL-13, IL-15, IL-16, IL-17 and IL-18 cytokines has resulted in a better understanding of immune responses during a pregnancy. NK and NK T cells and T cell subpopulation in endometrium and

peripheral blood as well as angiogenesis should also be considered when Th1/Th2 immune responses are considered.

Based on animal models, immunological reproductive failure in humans may not be the consequence of specific Th1/Th2 cytokine dysregulation, autoantibody abnormalities or NK cell abnormalities, but the reflection of a dysregulated immune response. Diagnosis and treatment of immunologically related reproductive failures such as recurrent pregnancy losses, infertility of implantation failures or preeclapmsia require not only immunological, but also genetic, endocrine and psychoneurological evaluation and tests. Animal models can provide some satisfactory explanations for the contradictory findings in the human experience that have led to strong disagreements among investigators. However, it is worth pointing out that verification of animal findings in humans is still a necessity.

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Relationship between Cytokine Concentration in Serum and Preovulatory Follicular Fluid and in vitro Fertilization/Intracytoplasmic Sperm Injection Outcome

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Abstract

Differences in follicle cell function and follicular fluid biochemistry have been suggested to influence the developmental potential of human oocytes at both cellular and chromosomal levels. Follicular fluids provide the environment in which oocyte maturation occurs. Therefore, follicular fluids may affect fertilization and early embryonic development. In humans, it has been shown that follicular fluid exerts chemotactic activity towards neutrophilic granulocytes and that the concentrations of this activity are related to the outcome of in vitro fertilization (IVF) treatment. The present study focuses on growth and cytokine presence in serum and follicular fluid of patients of reproductive age and their role in ovarian follicles, fertilization, embryo development, and implantation and to find out which growth factor could be a useful predictive parameter of IVF/intracytoplasmic sperm injection outcome.

Introduction

Immunocells and immunomodulatory cytokines have been shown to be active in several physiological processes in the reproductive tract [1]. The ovulatory process itself has been considered as a physiological inflammatory response, characterized by migration of leukocytes into ovulatory follicles. Besides, corpus

luteum formation and regression involve progressive infiltration of lymphocytes and macrophages, and communication through chemokines, cytokines, and cell adhesion molecules [2]. Additionally, monocytes and macrophages have been identified as the most abundant leukocyte subtype within the tissue of the human ovary [3, 4] and are also found in large numbers in human follicular fluid (FF) [3, 5]. Therefore, in the human, preovulatory FF has been shown to possess neutrophilic chemotactic activity [6, 7] which was higher in the ovulatory cycle than in the nonovulatory cycle.

Cytokines have been implicated as important regulators of steroidogenesis and gamete production [8, 9]. The microenvironment of human follicles is vital for normal oocyte development, folliculogenesis and ovulation. FF has been shown to improve the in vitro development of human preembryo and pregnancy rates [10, 11]. Gonadotropins are required to regulate this development, but growing evidence indicates that their effects are modulated by intraovarian factors [12, 13]. The growth, differentiation and apoptosis of ovarian follicular cells is regulated by the action and interactions of endocrine, paracrine and autocrine factors [14, 15]. In addition, many studies have revealed that various cytokines and growth factors play an essential role in preimplantation development [16, 17].

The temporal and spatial expression of several growth factors and cytokines within the uterus like interleukin-1 (IL-1) and its receptors [18], insulin-like growth factors I (IGF-I) and II (IGF-II) and their binding proteins [19], colony stimulating factors I [20], transforming growth factors-α (TGF-α) and β (TGF-β) [21, 22] and leukemia inhibitory factor (LIF) [23] suggest that they probably have an important functional role in uterine preparation for implantation [24]. The endometrial tissue is under control of steroid hormones, growth factors and cytokines [25]. Growth factors and cytokines have been suggested as a candidate important both for the communication between the endometrium and the blastocyst and for the implantation process [26, 27]. Moreover, the production and effect of cytokines at the implantation site are important for the regulation of trophoblast cell grow, differentiation and invasion [28, 29]. It is currently believed that for a normal development of pregnancy the production of inflammatory cytokines such as IL-2, tumor necrosis factor-α and interferon-γ is suppressed, whereas the production of anti-inflammatory cytokines such as IL-4, IL-6 or IL-10 is enhanced [30].

This study was undertaken in order to find out the presence of the most important growth factors and cytokines in serum and FF of patients of reproductive age and their effect on the ovarian function, fertilization, embryo development and implantation, and to determine which growth factor could be a useful predictive parameter of in vitro fertilization (IVF)/intracytoplasmic sperm injection (ICSI) outcome.

Insulin-Like Growth Factors

IGF are polypeptides which regulate growth, differentiation and survival in a multitude of cells and tissues [31]. Different studies point out that IGFs may play an important role in the human preovulatory process, and that IGF binding protein plays a key role in the regulation of follicular development by modulating IGFs and consequently gonadotropin action [32].

Concentrations of IGF-I were significantly higher ($p < 0.001$) in FF associated with mature oocytes that fertilized and cleaved than in FF associated with mature oocytes that did not fertilize [33]. Aritini et al. [34] found that in FF with mature oocytes IGF-I and GH concentrations were significantly higher compared to that of FF with atretic oocytes. They concluded that maturation of oocytes is associated with higher concentrations of GH, IGF-I and estradiol, but FF IGF-I and GH concentrations cannot serve as a predictor for IVF. Furthermore, addition of IGF-I to in vitro culture media has also been shown to benefit human embryo development [35], thereby increasing the blastulation rate from 35% in the control to 60% in the treatment group.

IGF-II found in trophoblast cells within the chorionic plate during lacunar villous stages may potentiate their invasive behavior [36] and stimulate insulin-like growth factor binding protein-1 (IGFBP-1) production by decidual cells in a paracrine manner [37]. Therefore, IGF-I, IGF-II and their binding protein have a vital role in implantation [38] and their effect on fetal development begins prior to implantation [39].

Heparin-Binding Epidermal Growth Factor

Heparin-binding epidermal growth factor (HB-EGF) is a member of the EGF family of growth factors which exert their action via EGF receptors. The mature form of HB-EGF is a potent stimulator of cell proliferation, migration and cell motility, while the transmembrane form of HB-EGF acts as a juxtacrine growth and adhesion factor [40]. The precise function of HB-EGF itself is still unclear. However, there is evidence that it can act as a paracrine factor to promote blastocyst growth in vitro, but the possibility that it induces changes in gene expression in the uterine epithelium and/or stroma associated with decidualization has not been explored [41]. In other studies, it has been shown that HB-EGF expression is elevated in the glandular and luminal epithelium during the late secretory (cycle days 20–24) phase [42, 43] when the human endometrium becomes receptive for blastocyst implantation [42]. Therefore, HB-EGF with its expression pattern appears to be highly relevant to the implantation process [44]. HB-EGF, calcitonin and endogenous cannabinoids were found to effectively

support peri-implantation blastocyst development [44, 45]. Besides, HB-EGF plays a role in both adhesion and development of the embryo [46].

Stavreus-Evers et al. [47] suggest that HB-EGF might play a role in both the attachment and penetration steps in the human implantation process and can be used as a marker for the implantation window. On the other hand, HB-EGF promotes human blastocyst growth and differentiation [48] and soluble HB-EGF is a potent growth factor for improving the development of in vitro fertilized human embryo into blastocysts and zona hatching [48]. Additionally, culture media supplementation with HB-EGF also improves the proportion of human embryo development capability as assessed by hatching, adherence to extracellular matrix proteins and trophectoderm outgrowth [48]. Ozornek et al. [49] demonstrated that EGF levels were significantly lower in patients where a pregnancy was established as compared to patients achieving no pregnancy ($p < 0.007$), which indicated that EGF is associated with IVF outcome.

Leukemia Inhibitory Factor

LIF, a pleiotropic cytokine, is expressed at low levels in many different tissues and exhibits a multitude of biological actions including modulation of proliferation and differentiation [50]. It is a member of the IL-6 cytokine family [51, 52]. It is possible that LIF could act on the hypothalamic-pituitary axis where LIF can influence hormone synthesis and therefore may indirectly affect ovarian and/or uterine function [53]. Ovarian granulosa cells, stromal cells and macrophages all express LIF mRNA and actively secrete protein [5]. LIF promotes the production of primary follicles in the ovary from primordial follicles [54].

Marked cyclical changes of LIF immunoreactivity in the human endometrial epithelium suggest a paracrine/autocrine role for LIF in endometrium function [55]. Many studies strongly suggest a critical role of LIF in the regulation of the physiological endometrial function [56, 57]. This cytokine is secreted throughout the menstrual cycle and its expression is high in progesterone-dominated implantation stage endometrium [58, 59].

However, LIF is not directly regulated by steroid hormones in the human endometrium [60, 61]. Endometrial LIF expression is dependent on cellular localizations and menstrual stages. Stronger LIF expression presents in the endometrial epithelium during the luteal phase [62]. LIF protein is normally produced by the endometrium and several subsequent studies have shown that it is involved in the control of implantation [63, 64]. Endometrial LIF expression is related to human fertility. In fertile women, LIF mRNA expression is observed during the secretory/postovulatory phase of the menstrual cycle [23, 55].

Maximal expression of LIF was found between days 19 and 25 of the menstrual cycle, coinciding with the time of human implantation [60, 64]. Treatment of mice leading to increased LIF mRNA values in the uterus promotes embryo implantation [65], whereas targeted deletions of LIF genes resulted in infertility [66]. Moreover, LIF has been found in uterine flushing during the time of expected implantation in fertile women [67]. Uterine flushing taken from infertile women during the secretory phase contains lower levels of LIF protein when compared with fertile controls [67]. LIF production in fertile women on days 18–21 of the menstrual cycle was 3.5 times higher than in infertile women with multiple failures of implantation and 2.2 times higher than in infertile women without failure of implantation [68].

In vitro studies showed that endometrial explants taken from infertile women secrete less LIF than biopsies taken from fertile patients [68, 69]. However, biopsies cultured in serum-free culture mediums showed no significant difference in LIF secretion between fertile and infertile patients [70]. Secretion of LIF was lower in cultures of human endometrial explants from women with repeated failure to implant and from women with unexplained infertility [23, 69, 71]. LIF is also implicated in human implantation; inactivation of gp130, a signalling partner for the LIF receptor, also results in implantation failure [27, 72]. Besides, LIF appears to be important for both decidualization and implantation [23, 73]. Cullinan et al. [23] showed that endometrial LIF influenced blastocyst implantation through autocrine-paracrine interaction at the luminal epithelium level and blastocyst stage. Blastocysts also express the heterodimeric LIF receptor [74].

In humans, FF LIF has been described as a marker of oocyte quality [75]. The human FF LIF concentrations increase upon HCG administration and correlated with embryo quality [76]. It has also been reported to enhance the rate of blastocyst formation [63]. Lower concentrations of LIF FF were found in the polycystic ovary patients and are most likely related to the lower implantation rate, since FF LIF appears to function as an embryotrophic agent [77]. Studies in a mouse model suggest that LIF may have an embryotrophic effect [78] but supplementation of culture media with recombinant human LIF did not enhance human blastocyst formation in vitro [79].

Addition of LIF to a complex serum human embryo culture medium increased the blastulation rate from 18 to 44% [63]. However, Jurisicova et al. [79] did not find such an effect. Olivennes et al. [80] indicated that LIF was detected in uterine flushing in 46% of patients at the time of egg retrieval, but did not indicate any correlation with better pregnancy rates. LIF concentrations were similar in patients establishing a pregnancy as compared to those who did not [49]. Wu et al. [81] could not find a relationship between serum LIF levels and pregnancy outcomes in their IVF patients.

Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a heparin-binding homodimeric glycoprotein of 30–40 kDa that is produced by many tissues including the endometrium and ovary acting as an angiogenic factor important for neovascularization of the reproductive tract [82]. In the ovary, VEGF is produced both by granulosa and thecal cells [83, 84]. It has been suggested that VEGF is associated with follicles showing high vascularization and oxygenation resulting in oocytes with superior pregnancy potential [85, 86]. VEGF is also involved in many processes related to reproductive physiology, including angiogenesis, which is essential for implantation and placentation [87, 88].

Several studies have demonstrated that VEGF is produced by granulosa cells and released into FF after gonadotrophin stimulation [89, 90]. VEGF may play an important role in the regulation of vascular development during follicular growth and luteal differentiation [91]. In patients undergoing IVF, FF VEGF levels at the time of egg retrieval correlated with the degree of follicular luteinization. There is a significant ovarian contribution to circulating VEGF levels during early gestation [92]. Elevated VEGF levels appear to be markers of follicular hypoxia and suboptimal embryo development [93].

A positive correlation has been observed between FF VEGF concentrations and patients' age, especially in patients ≥38 years of age undergoing IVF [94, 95]. Friedman et al. [94] investigated whether differences in FF VEGF concentrations are observed between women achieving a clinical pregnancy and those failing to conceive. They found that elevated FF VEGF concentrations are associated with poor conception rates after IVF or gamete intrafallopian transfer (GIFT).

Manau et al. [95] investigated FF and circulating concentrations of adrenomedullin, VEGF and nitric oxide in 70 IVF patients. They found that neither serum nor FF concentrations of adrenomedullin, VEGF or nitrite/nitrate were correlated with IVF outcome.

Benifla et al. [96] showed that there was no statistically significant difference in the FF VEGF concentrations in patients conceiving and those failing to conceive. This suggests that VEGF production by granulosa cells is not directly correlated with assisted reproduction outcome or pregnancy potential. The serum and FF VEGF levels were found to be significantly lower in the group in whom the pregnancy was achieved [97]. It is proposed that the circulating VEGF in pregnancy is derived from the decidual and trophoblast tissues [98]. Clark and Kamen [99] and Shiraishi et al. [100] demonstrated by immunohistochemical staining that VEGF synthesized of cytotrophoblast and extravillous trophoblast cells in early pregnancy.

On the other hand, VEGF has been implicated in the pathogenesis of capillary leakage, which in turn is a major mechanism of ovarian hyperstimulation

syndrome, a severe complication of IVF procedures [101, 102]. VEGF levels have a high predictive value in differentiating the various types of pregnancies like ectopic pregnancy, first-trimester miscarriage and biochemical pregnancy [103–105].

Transforming Growth Factor

TGF-β is represented by 5 homodimeric polypeptides which share 70–80% structural homology. TGF-β proteins and mRNA have been identified in endometrial stromal, epithelial and decidual cells [106, 107]. TGF-α have been localized in the endometrium during the proliferative and the secretory phase, but its expression is particularly high in decidual cells [108].

TGF-α is indiscriminately expressed in the peri-implantation mouse uterus and its role in implantation is questionable because TGF-α null mice are apparently fertile [109, 110].

Fried and Wramsby [111] showed that FF and serum concentration values of 29 women who became pregnant following IVF were significantly higher ($p = 0.005$) than in 59 women where IVF was unsuccessful. Therefore, TGF-β1 may be important for successful human preembryo development, contribute to successful embryo implantation and development and may be necessary for the establishment of pregnancy.

Basic Fibroblast Growth Factor

Fibroblast growth factor (FGF) stimulates ovarian granulosa cell differentiation [112], expression of LH receptors by granulosa cells, and proliferation of ovarian germinal cells [113].

Basic FGF (10 ng/ml) decreased the rate of blastocyst formation and murine embryo hatching. The level of basic FGF did not change in the FF around ovulation in women undergoing ovarian hyperstimulation for IVF therapy and there was no correlation between FF basic FGF levels and reproductive parameters with the exception of age [114]. No correlation could be found between ICSI outcome and FGF concentrations in serum or FF [115].

Tumor Necrosis Factor

Tumor necrosis factor (TNF) is a pleiotropic cytokine 14-kDa polypeptide produced by several cell types and particularly by macrophages [17]. TNF-α

exerts cytotoxic as well as differentiation and growth modulator activities on many different target cells [116]. TNF-α activates inflammatory leukocytes, resulting in the production of other proinflammatory cytokines such as IL-1, IL-6 and IL-8 [117].

INF is a cytokine with an essential role in folliculogenesis and ovarian maturation [118]. It is a potent modulator of ovarian function, affecting steroidogenesis of both granulosa and interstitial thecal cells [119]. The precise role of TNF-α in the regulation of granulosa cell fate beyond these follicular stages is not known. Moreover, TNF-α and its mRNA are expressed by epithelial, stromal and lymphoid cells of the human endometrium [120, 121]. Philippaeaux and Piguet [122] showed that the level of TNF-α increases towards the late secretory phase of the menstrual cycle. In early pregnancy when trophoblast invades the endometrium TNF-α inhibit the trophoblast proliferation [123] and TNF receptors have been characterized on human trophoblast cells [123]. Additionally, TNF induces MMP-1 and MMP-3 in human chorionic cells [124]. The serum concentration of TNF-α shows significant fluctuations during the menstrual cycle. The concentrations are significantly increased during the late follicular phase and during mid-luteal phase. In the early luteal phase, the values are significantly decreased [125]. TNF-α concentrations in FF were significantly higher, in poor quality oocytes ($p < 0.001$), but not associated with infertility-associated diseases and the oocyte maturity [126].

Granulocyte-Macrophage Colony-Stimulating Factor

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is not only a product of activated T lymphocytes, it is now known to be produced by a divers array of hemopoietic and nonhemopoietic cell lineage, including macrophages, mast cells, fibroblast, endothelial cells and epithelial cells [127]. GM-CSF is expressed in several reproductive tract tissues including endometrium of human and other species [128, 129].

The uterine epithelium has been identified by in situ hybridization and in vitro cell isolation studies as the major source of GM-CSF in both the cycling and pregnant mouse uterus [1] and in the ovine uterus [128]. A critical role for GM-CSF in normal development of human embryos has been determined.

Frozen-thawed embryos cultured in the presence of the recombinant GM-CSF have a significantly increased cleavage rate which is known to be a sign of high developmental potential [130]. The addition of this cytokine to embryo culture media may improve the yield of implantation-competent blastocysts in human IVF programs [131]. However, in our study, Hammadeh et al. [132] found no significant difference in GM-CSF levels between

patients who became pregnant after ICSI treatment and those who did not ($p = 0.12$).

Colony-Stimulating Factor-1

Colony-stimulating factor-1 (CSF-1), a hematopoietic growth factor, supports the growth and proliferation of mononuclear progenitor cells and promotes the proliferation of mature macrophages [133].

The presence of CSF-1, a primary regulator of tissue macrophages in FF and the presence of mRNA for CSF-1 and its receptor c-fms in FF-derived cells, suggests a role for this growth factor in ovarian function [134]. However, CSF-1 mRNAs are expressed in human endometrial glands during the midproliferative and midsecretory phase of the menstrual cycle [16]. A correlation between abnormal CSF-1 expression and infertility has not been established. Pollard et al. [135] suggested that CSF-1 has an important role in successful pregnancy.

FF concentration of CSF-1 at oocytes retrieval after ovarian stimulation (median 3.116 pg/ml, range 1.824–5.883 pg/ml) was about 7-fold higher than blood concentrations (median 472 pg/ml, range 250–1,055 pg/ml; $p < 0.0001$) which suggest that the intraovarian CSF-1, possibly induced by LH/HCG, plays an important role during ovulation and luteinization [136]. CSF-1 concentration in serum increased during ovarian stimulation and reached the highest concentrations during the mid-luteal phase, independently of the outcome of treatment [137]. Witt and Pollard [134] reported that immunoreactive CSF-1 concentrations in FF were 2.8-fold higher than those in serum. Another study [136] showed that the concentrations of CSF-1 in FF obtained from dominant follicles during the menstrual cycle and ovarian stimulation were about 4- and 7-fold higher than those in plasma at the same time, respectively.

Granulosa Colony-Stimulating Factor

Granulosa colony-stimulating factor (G-CSF) is a pleiotropic cytokine and is produced primarily by hematopoietic cells, endothelial and epithelial cells, as well as reproductive tissue cells [138, 139].

CSF-1, known as macrophage colony-stimulating factor (M-CSF-1), is a glycoprotein that belongs to the family of hematopoietic growth factor and is primarily responsible for proliferation, differentiation and survival of the monocyte-macrophage cell lineage [99, 140]. G-CSF and its receptor produced by placental, decidual and endometrial gland cells during pregnancy indicate that G-CSF may play a role in decidual and placental functions [141, 142]. -M-CSF acts as a

regulator of placenta [143, 144] or trophoblast growth and differentiation [145] and M-CSF has also been suggested as having a role in fetal development.

Yanagi et al. [146] described the presence and local production of G-CSF in the human ovary and found the G-CSF produced mainly by granulosa cells, theca, and stromal cells. Hock et al. [147] reported that in ovarian-stimulated patients, the white blood cell counts and G-CSF level in serum rose significantly during the stimulation cycles. G-CSF concentration increases significantly during the ovulatory phase compared with other phases, suggesting that G-CSF may play an important role in ovulation [148]. G-CSF was found in high concentrations in FF and in serum on the day of oocyte retrieval. However, the significantly higher level of G-CSF in FF than in serum and the expression of G-CSF and its receptor by granulose cells suggest an important role for this growth factor in ovarian function [149].

Several reports have indicated that M-CSF [143] and monocyte chemoattractant protein-1 (MCP-1) [76] are present in human FF and they might be implicated in ovulation. But their biological role and effect on the oocytes is still not known in detail. Kawano et al. [150] reported that the concentrations of M-CSF and MCP-1 were higher in FF containing mature oocytes than in those containing immature human oocytes and the concentrations in FF were higher than in serum at the time of oocyte retrieval.

Kawano et al. [150] suggest that M-CSF and MCP-1 may play an important role in preovulatory processes and that M-CSF, in particular, may be regulated by cyclic adenosine monophosphate. M-CSF and MCP-1 may also be a valuable biochemical marker in the process of oocyte maturation.

Stem Cell Factor

KL (also termed stem cell factor, mast cell factor or steel factor) has a wide range of activities on germ cells, melanocytes, mast cells and primitive hematopoietic cells of the myeloid, erythroid, and lymphoid cell lineage [150–152].

The Kit ligand/stem cell factor (KL) appears to be one of the first factors identified to be involved in the promotion of primordial follicle development. Parrott and Skinner [153] suggested that KL is necessary and sufficient to induce primordial follicle development and initiate folliculogenesis. Horie et al. [154] showed the presence of c-kit in the human oocytes. Laitinen et al. [155] found that stem cell factor is produced by the cumulus granulosa cells and that levels are regulated by FSH, suggesting that stem cell factor may be important in human follicular development. Bedell et al. [156] demonstrated that DNA rearrangements of the promoter region for stem cell factor also disrupt follicular development.

Stem cell factor may play a role in human follicular and oocyte development, and increasing intrafollicular stem cell factor concentrations may improve fertilization and embryo transfer after oocyte retrieval. Consequently, elevated FF stem cell factor concentrations are associated with an increased likelihood of IVF success [157]. Hammadeh et al. [115] found SCF concentrations either in FF or in serum were similar between patients who became pregnant after ICSI and those who did not.

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Immunocontraceptive Approaches in Females

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Abstract

In the female reproductive system, there are multiple points, where immunointerception is feasible. Among these, the most promising are those that interfere immunologically at the level of sperm-egg interaction or neutralize human chorionic gonadotropin (hCG), which is required for the establishment and maintenance of pregnancy. Immunization studies in various animal models including non-human primates with zona pellucida (ZP) glycoproteins have demonstrated curtailment of fertility. To circumvent the ovarian pathology often observed following immunization with ZP glycoproteins, synthetic peptides devoid of 'oophoritogenic' T cell epitopes as immunogens have been proposed. Though of utility for controlling wildlife populations, its application in humans will have to wait till the safety of ZP glycoprotein vaccine is established beyond doubt. Clinical trials in women have established that immunocontraceptive vaccine based on β-subunit of hCG inhibits fertility. To make it a practical proposition, novel strategies have to be adopted to increase its immunogenicity so that 'contraceptive' antibody titers are achieved in near to 100% recipients.

Introduction

It is projected that by the year 2050, the global human population may reach 10 billion. Of this, a major increase in the human population is likely to occur in third world countries, which will further impose a major challenge to provide basic amenities such as food, education, employment and health care. This necessitates the judicious and effective use of currently available methods of contraception such as oral pills, intrauterine devices, hormonal implants,

condoms and terminal methods like vasectomy for males and tubectomy for females to stabilize the human population, if possible, by the year 2025. Based on feedback from the end users and taking into account the recent scientific developments, these products/technologies are being further refined to make them more user-friendly and efficacious. Vaccines, which have been effectively used to control several infectious diseases, are being explored as a tool for contraception and termed as immunocontraceptive vaccines. It involves generating humoral and/or cell-mediated immune responses against antigens that are critical in reproductive processes and, therefore, interference in their biological function will result in a block of fertility. A block in fertility by immunocontraceptive vaccines can either be reversible, in which case fertility is regained subsequent to a decline in antibody response, or irreversible as evident by a failure to regain fertility in spite of undetectable circulatory antibodies. An ideal immunocontraceptive vaccine for humans should be (1) effective in generating an adequate immune response in 100% of the recipients, (2) potentially reversible, (3) free of any side effects, and (4) cost effective. In this article, we review the various approaches being used to develop immunocontraceptive vaccines as applicable in females.

Targets for Immunological Intervention in the Female Reproductive System

The gonadotropin-releasing hormone (GnRH) secreted by the hypothalamus acts on the pituitary and regulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH). The LH and FSH, in turn, act on the ovaries wherein these hormones regulate the follicular growth, maturation of oocytes and ovulation. Neutralization of GnRH biological activity by generating a specific antibody response will interfere in the production and maturation of oocytes, thereby leading to a block of fertility. One of the promising targets for immunological intervention may be at the level of sperm-egg interaction. Both male and female gametes have unique surface determinants against which an immune response can be elicited, thereby leading to a failure of fertilization per se. Subsequent to fertilization, the one-cell embryo starts growing and secretes human chorionic gonadotropin (hCG). The circulating hCG helps in the rescue of corpus luteum function and, thus, makes possible the establishment and maintenance of pregnancy. Immunological neutralization of hCG by antibodies can lead to a failure of implantation of the blastocyst. In the female reproductive system, thus, there are multiple targets, which can be used for the development of immunocontraceptive vaccines.

Immunological Intervention at Prefertilization Stage

Gonadotropin-Releasing Hormone

Generation of antibodies against GnRH, a decapeptide hormone, may have wide-ranging consequences leading to a block in the secretion of pituitary gonadotropins and gametogenesis and, hence, may not be an acceptable proposition for fertility inhibition in humans. Nonetheless, immunoneutralization of GnRH by passive or active immunization leads to a block of fertility in various animal species [1–3]. Active immunization of white-tailed female deers *(Odocoileus virginianus)* with GnRH-based immunocontraceptive vaccine led to an 88% reduction in fawning rates, altered estrus behavior, reduced concentrations of progesterone and a failure to maintain pregnancy following conception [4]. In this study, GnRH vaccine induced reversible infertility lasting for 2 years without boosting. Another application of GnRH-based vaccine, which may find favor, is to perform immunocastration of young pigs thereby providing a humane alternative to surgical castration [5].

Sperm-Specific Antigens

In women, several clinical studies have revealed that antibodies against sperm are frequently associated with otherwise unexplained infertility [6]. These experiments of nature documenting that immunocontraception is feasible in humans prompted several groups to identify, characterize and evaluate the immunocontraceptive potential of spermatozoa-specific antigens.

Zona pellucida Glycoproteins

During fertilization, zona pellucida (ZP), an acellular matrix that surrounds the mammalian oocyte, serves as the 'docking' site for species-specific recognition and binding of the spermatozoa to the oocyte. In addition, it induces acrosome reaction in the ZP-bound spermatozoa, affects avoidance of polyspermy and protects the growing blastocysts before implantation. The ZP is mainly composed of three sulfated glycoproteins, designated as ZPA, ZPB and ZPC based on the size of their transcripts.

Active Immunization with ZP Glycoproteins

By virtue of the fact that ZP glycoproteins play a critical role in successful fertilization, these have become promising candidate antigens for the development of an immunocontraceptive vaccine. For this purpose, various forms of ZP glycoproteins have been employed, which are described below.

ZP Glycoproteins from Native Source

Comparison of deduced amino acid (aa) sequence of ZPA, ZPB and ZPC characterized from various species revealed that there is a variable degree of conservation. Due to this, antibodies generated against ZP glycoproteins from a given species react to a variable extent with the ZP from other species and permit heterologous immunization. An early study demonstrated that immunization of mice with heat-solubilized hamster zonae led to a block in fertility [7]. Infertility was also observed in female rabbits as well as nonhuman primates immunized with heat-solubilized porcine ZP [8, 9]. The block in fertility in both species was accompanied by follicular atresia and an abnormal hormonal profile and was not due to inhibition of fertilization. At this juncture, it was construed that the contamination of heat-solubilized ZP by other ovarian-associated proteins may be responsible for the observed side effects on ovaries.

Subsequently, various groups made efforts to purify different ZP glycoproteins and employ these to determine the immunocontraceptive potential and safety of such a procedure. Infertility was observed in squirrel monkeys *(Scimiri sciureus)* immunized with purified porcine ZP3 (a mixture of porcine ZPB and ZPC) [10]. During the initial phase of immunization, disturbances in the secretion of ovarian steroid hormones and folliculogenesis were observed, which returned to normal 10–15 months postimmunization. Studies by our group revealed that female bonnet monkeys *(Macaca radiata)* immunized with purified porcine ZP3, employing adjuvants permissible for human use, in spite of ovulatory cycles, failed to conceive in the presence of high circulating antibody titers [11]. Laparoscopic examination revealed normal ovaries with developing follicles. Following a decline in the antibody titers, 50% of the animals became pregnant. Ovarian histology of the animals that failed to regain fertility did not reveal any signs of inflammation or lymphocytic proliferation. There was also no observed increase in the number of atretic or degenerating follicles. These studies suggest that employing a highly purified form of ZP glycoproteins as immunogens can minimize the adverse effects on ovarian functions.

Recombinant ZP Glycoproteins

The use of recombinant proteins may circumvent the problems associated with immunization by ZP glycoproteins isolated from a native source that may be contaminated with the other ovarian-associated proteins. Long-term infertility was observed in female marmosets *(Callithrix jacchus)* immunized with human ZPC expressed in mammalian cells [12]. Observed infertility was due to depletion of the primordial follicle pool. In another study, female cynomolgus monkeys *(Macaca fascicularis)* and baboons

(Papio cynocephalus) were immunized with recombinant human ZPA, ZPB and ZPC expressed in Chinese hamster ovarian cells. Long-term infertility (9–35 months) was observed in animals immunized with ZPA [13]. During the time of high antibody titers, some animals experienced disruption of the menstrual cycles, which eventually returned to normal. Studies by our group revealed that immunization of female baboons *(Papio anubis)* with recombinant bonnet monkey ZPB (r-bmZPB) conjugated to diphtheria toxoid (DT) also led to a block of fertility, which was reversible [14]. In contrast, immunization of female bonnet monkeys with r-bmZPB or recombinant bonnet monkey ZPA (r-bmZPA) conjugated to DT led to a block in fertility, which is irreversible [15]. Ovarian histopathology of the immunized animals revealed the presence of atretic follicles and degenerated oocytes, which may have been the principle cause for the block in fertility.

ZP Glycoprotein-Based Live Recombinant Vectors

With the hope of increasing immunogenicity, live recombinant vectors encoding ZP glycoproteins have been constructed. Oral immunization of female mice with recombinant Salmonella expressing mouse ZPC generated IgG antibodies in vaginal secretion against ZP and led to partial inhibition of fertility [16]. In addition, the potential of ectromelia virus, a natural pathogen for mice that causes mouse pox and murine cytomegalovirus as live recombinant vector expressing mouse ZPC, has also been evaluated [17, 18]. In both cases, immunization of female mice with recombinant virus resulted in a curtailment of fertility.

ZP Glycoprotein-Based DNA Vaccine

Another alternative approach to conventional immunization with proteins is to use plasmid DNA encoding the said protein. The efficacy of DNA vaccine has been demonstrated for a variety of bacterial, viral and protozoan infections [19]. Our group has cloned the cDNA encoding bmZPB and dog ZPC in mammalian expression vector VR1020, downstream of a tissue plasminogen activator signal sequence under a cytomegalovirus promoter [20, 21]. Immunization of mice with the above plasmid DNA constructs induced antibodies reactive with the native ZP. Interestingly, antibodies thus generated by immunization with plasmid DNA encoding bmZPB also inhibited in vitro binding of human spermatozoa to human ZP in a hemizona assay [20]. In another study, immunization of mice with the plasmid DNA encoding partial sequence of rabbit ZPC (aa residues 263–415) led to inhibition of fertility [22]. Interestingly, infertility was not associated with any interference in follicular development.

ZP Glycoprotein-Based Synthetic Peptides

Keeping in view the observed ovarian dysfunction, in spite of using recombinant ZP glycoproteins, which are devoid of other ovarian-associated proteins as a contaminant, prompted us to suggest that there is something within the zona proteins that may be responsible for these side effects. This notion was confirmed by performing a series of experiments, which suggested the involvement of 'oophoritogenic' T cell epitopes within the zona proteins [23]. Subsequently, efforts were made by several groups to delineate B cell epitopes of the three ZP glycoproteins that are devoid of 'oophoritogenic' T cell epitopes [24–28]. Murine polyclonal antibodies generated against chimeric peptide encompassing the bmZPC epitope (334–343 aa) synthesized colinearly with a 'promiscuous' T cell epitope of circumsporozoite protein of *Plasmodium falciparum* (378–398 aa) inhibited in vitro the binding of human sperm to human zona [24]. Similar results were obtained with the polyclonal antibodies generated in cynomolgous monkey against chimeric peptide encompassing monkey ZPC (334–342 aa) and *P. falciparum* T cell epitope [25]. In addition to ZPC, polyclonal antibodies against hZPA have also shown in vitro contraceptive potential [26, 27]. Immunization of mice with mouse ZPA peptide (121–140 aa) led to a generation of antibodies that recognized native ZP. No adverse effects on the ovaries were observed in the immunized mice [28].

Immunization of mice of eight different haplotypes and B6AF1 mice with chimeric peptide comprising of 'promiscuous' T cell epitope of bovine RNase (NCAYKTTQANK) colinearly synthesized with the minimal B cell epitope of mouse ZPC (335–342 aa; Phe_{336} substituted with Ala) led to generation of antibodies against mouse ZPC and decrease of fertility, which was associated with antibody titers [29]. No oophoritis was observed in the immunized mice. Similarly, no disruption of the ovarian function was observed in female marmosets immunized with the synthetic peptides corresponding to human or marmoset ZPC [12, 30]. However, immunization with the synthetic peptide did not lead to a consistent reduction in fertility. In another study, female bonnet monkeys immunized with bmZPC synthetic peptide (324–347 aa) conjugated to DT failed to conceive when mated with males of proven fertility [31]. The immunized animals exhibited normal ovulatory cycles in spite of high circulating antibody titers against the bmZPC peptide and immunization was not accompanied with any ovarian pathology.

To enhance the immunogenicity of the ZP-based synthetic peptide immunocontraceptive vaccine, we have proposed to use 'multiple' B cell epitopes either as physical mixture [32] or assembled in a single construct [33]. Antibodies generated against a recombinant chimeric protein encompassing bmZPB (132–147 aa), bmZPA (86–113 aa) and bmZPC (324–347 aa) B cell

epitopes conjugated to DT effectively inhibited in vitro the binding of human sperm to human zona [33]. It will be of interest to evaluate the in vivo efficacy of such a chimeric recombinant protein by undertaking active immunization studies. Another elegant approach to design an effective immunocontraceptive vaccine to inhibit fertility may be to employ chimeric recombinant protein comprising of ZP and spermatozoa antigens [34].

Immunological Intervention at Postfertilization Stage

The hCG is a favored candidate for development of an immunocontraceptive vaccine for females by virtue of the fact that it is synthesized postfertilization and is adjudged crucial for the establishment and maintenance of pregnancy at least during the first 7–9 weeks of gestation. After carrying out extensive immunogenicity, safety and toxicological studies in a variety of animal models of vaccine comprised of β-hCG linked to tetanus toxoid (TT), the group of Talwar et al. [35] in India conducted phase I clinical pharmacological trials on 63 women in India, Helsinki, Uppsala, Santiago and Bahia. This study revealed that immunization led to generation of anti-hCG and anti-TT antibodies. However, the antibody titers and duration of the immune response were variable among immunized women. No clinical or endocrine abnormalities were observed during these trials, thus demonstrating the safety of the procedure and lack of side effects [36]. Subsequently, this group after extensive research input modified the vaccine formulation to make it more immunogenic. In landmark extended phase II clinical trials of the vaccine comprised of a heterospecies dimer of β-hCG annealed with the α-subunit of ovine luteinizing hormone (α-oLH) coupled to either TT or DT, this group demonstrated that immunization of women led to generation of anti-hCG antibodies that neutralized the bioactivity of hCG [37]. Immunized women having circulating bioneutralizing antibody titers above 50 ng/ml were protected against conception. Only one pregnancy was observed out of 1,224 cycles. However, an antibody titer above 50 ng/ml was observed only in 80% of the immunized women. Currently, efforts are ongoing to improve the immunogenicity of the β-hCG-based vaccine by incorporating more potent adjuvants and using the 'promiscuous' T non-B cell epitopes as carrier proteins instead of DT/TT.

Another alternative approach, supported by the World Health Organization and developed by Stevens and coworkers [38], employed a 37-amino acid carboxy-terminal peptide (109–145 aa) of β-hCG as it is unique to hCG and its absence in LH, FSH and TSH may lead to generation of a more specific antibody response. Immunization of female baboons with the peptide linked to DT along with muramyl dipeptide adjuvant and emulsified with squalene and mannide

monooleate reduced the fertility rate considerably. In some of the immunized animals, autoantibodies against pancreas and pituitary were observed. Subsequently, phase I clinical trials in 30 young healthy sterilized (by tubal ligation) women in Bedford Park, Australia revealed that immunization with this vaccine formulation led to generation of potentially contraceptive levels of antibodies to hCG in all immunized subjects [39]. The phase II trials employing this formulation to evaluate its efficacy to prevent conception were abandoned due to observed adverse local reaction at the site of injection in some volunteers.

In order to minimize the cross-reactivity with LH of antibodies generated against β-hCG-based vaccines, analogs of β-hCG prepared by employing site-directed mutagenesis, whereby LH cross-reactive epitopes have largely been eliminated, have also been used [40, 41]. Immunization of mice and rabbits with such mutant β-hCG generated antibodies, which have low cross-reactivity with LH [41, 42]. Recently, the immunogenicity of the β-hCG-based DNA vaccine has also been demonstrated [43]. Cloning of β-hCG along with C3d adjuvant led to generation of a 9-fold higher antibody response as compared to β-hCG alone. The DNA vaccination decreases the secretion of Th1 cytokines (IL-2, TNF-γ and TNF-α) and increases the expression of Th2 cytokines (IL-4 and IL-10) in response to hCG antigen.

Riboflavin-carrier protein (RCP), in mammals, is a major transporter of vitamin to the developing embryo across the placental barrier. The potential of using RCP as a candidate antigen for development of immunocontraceptive vaccine has been extensively studied by scientists at the Indian Institute of Science, Bangalore. Active immunization of female rodents and monkeys with linearized denatured RCP significantly reduces fertility [44]. Subsequently, these investigators have also demonstrated the potential of the synthetic peptides pertaining to linear B cell epitopes in curtailing pregnancy in rodents and subhuman primates [45]. Further work is required to validate RCP-based contraceptive vaccine as a viable proposition.

Concluding Comments

There is a need to undertake long-term active immunization studies with the currently pursued contraceptive vaccines, in particular, based on ZP to establish their safety in an unambiguous manner, before these can be considered for human application. Nonetheless, ZP-based contraceptive vaccines have a bright future for controlling wildlife populations [46–48]. Another major challenge will be to increase the immunogenicity of contraceptive vaccines so as to generate 'contraceptive' antibody levels in 100% of the recipients. An alternate route of vaccine delivery, such as the oral route, will also constitute an important component in

this endeavor. Basic research inputs will be required to identify and characterize novel target candidates that are crucial for gametogenesis, fertilization and implantation, which may serve as additional candidate antigens for the development of more effective and safer contraceptive vaccines.

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Pregnancy-Associated Immunoregulatory Molecules Discovered in Ruminants and Their Possible Relevance to Other Species

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Abstract

Several distinct molecules involved in maternal-conceptus interactions have been discovered in ruminants. Among these are two families of immunoregulatory molecules that represent genes that have undergone evolution to perform a function distinct from that of the ancestral gene. Interferon-τ (IFN-τ) is a product of the trophoblast that retains its antiviral activity and other functions characteristic of interferons but whose primary role is the inhibition of luteolysis. The uterine serpins are progesterone-induced members of the serpin superfamily of serine proteinase inhibitors. The uterine serpin has only a weak antiproteinase activity and, at least in the sheep, appears to function as an inhibitor of lymphocyte proliferation to mediate the immunosuppressive effects of progesterone on uterine immune function. The IFN-τ are not present in mammals that diverged from ancestors of ruminants before 36 million years ago, the approximate origin of IFN-τ, but uterine serpins apparently arose before the divergence of mammals and the proteins are present in at least one nonruminant species, the pig. The clinical value of these molecules can extend beyond use in ruminants. IFN-τ has been used to treat experimental allergic encephalomyelitis in mice and can inhibit human immunodeficiency virus replication. Ovine uterine serpin is also active against murine and human cells but a clinical use has not yet been identified.

Introduction

The pecoran ruminants diverged as a separate infraorder during the Middle Eocene epoch about 33–46 million years ago and radiated into 5 separate families during the Early/Late Olicene transition about 20–29 million years ago

[1]. While the basic pattern of reproduction in pecoran ruminants is similar to other mammals, there are distinct features of reproduction including, most prominently, characteristics of placental anatomy [2]. There are also several molecules involved in endocrine and paracrine signaling processes during pregnancy that have been described in pecoran ruminants only.

Despite the unique features of pecoran reproduction, the immunological relationship between the conceptus and mother is similar in many ways to that of other mammals. The uterus of ruminants contains components of the immune system capable of tissue graft rejection responses [3, 4]. Like for other species, expression of MHC antigens on the trophoblast is largely downregulated [5, 6] although, at least in the cow, there is limited expression of class I MHC molecules on trophoblast in later pregnancy and the degree of fetal-maternal MHC compatibility affects placentomal lymphocyte populations and cytokine expression [6]. That extensive regulation of lymphocyte populations in the uterus takes place is indicated by the dynamic changes in uterine lymphocyte populations that occur during pregnancy. In the sheep, for example, macrophages accumulate in large numbers in the endometrial stroma during pregnancy [7] and a population of granulated γδ T cells becomes abundant in luminal epithelium of the interplacentomal regions during mid and late pregnancy [8, 9]. In contrast, numbers of nongranulated T cells in the glandular epithelium decline during pregnancy while numbers of these cells in luminal epithelium first decline and then return to levels seen in nonpregnant ewes [8, 9]. T cells are nearly absent in the placentomes [5].

The nature of the regulatory signals controlling changes in lymphocyte function during pregnancy is incompletely understood. Studies in which pregnancy is confined to one uterine horn have provided evidence for both systemic and local regulation of endometrial leukocyte populations [7, 9]. Both placental and endometrial tissues also produce a variety of cytokines and growth factors. For the purposes of this review, focus is placed on the properties of two immunoregulatory molecules that were initially discovered in ruminants and which appear to represent genes that have undergone evolution to perform a function distinct from that of the ancestral gene. Interferon-τ (IFN-τ) is a product of the trophoblast whose synthesis peaks around the time when placental attachment begins. While it retains its antiviral activity and other functions characteristic of interferons, the IFN-τ gene has evolved to encode for a protein whose primary role is the inhibition of luteolysis to maintain sustained progesterone secretion [10]. The other protein, uterine serpin, is a progesterone-induced member of the serpin superfamily of serine proteinase inhibitors. Uterine serpin has only weak antiproteinase activity and appears to function as an inhibitor of lymphocyte proliferation to mediate the immunosuppressive effects of progesterone on uterine immune function [11].

Interferon-τ

The IFN-τ gene arose via gene duplication from IFN-ω about 36 million years ago [12], a time before the radiation of the pecoran ruminants about 20–29 million years ago [1]. Thus, IFN-τ genes have been found in a variety of pecoran ruminants including the cow, sheep, goat, musk ox, and giraffe. The genes continued to evolve after the initial gene duplication – 12 genes have been identified in sheep and 9 in cattle.

As pointed out by Roberts et al. [12], it is not its activity that makes IFN-τ such an important molecule in maintaining corpus luteum function because other interferons can also block luteolysis [13] and because IFN-τ retains the antiviral, antiproliferative, natural killer (NK) cell-activating, and immunosuppressive properties of type I interferons [14–18]. Rather it is its unique tissue-specific and temporal expression pattern. In particular, IFN-τ gene expression is limited to the trophoblast for only a few days at a time coincident with the time when the uterus is poised to produce prostaglandin-$F_{2\alpha}$ ($PGF_{2\alpha}$) pulses that lead to luteolysis. In the cow, for example, the trophoblast produces low amounts of IFN-τ beginning at the expanded blastocyst stage at ~day 8 of pregnancy [19]. Synthesis becomes prominent by days 12–13, peaks at days 15–17, and continues to be produced in decreasing amounts until it ceases between day 25 and 29 [20–22]. These times correspond to the programmed time for luteolytic release of $PGF_{2\alpha}$ at days 16–19 after estrus [23]. The evolutionary changes that lead to this pattern of gene expression must therefore have involved changes in the transcriptional control of IFN-τ gene expression. IFN-τ genes have no apparent viral control elements in the promoter region but do contain an Ets-2 binding site that is likely required for expression in the trophoblast [12]. Signals from the uterus are important for induction of IFN-τ gene expression; expression can be induced by placing in vitro derived embryos in the uterus for several days [19]. The specific uterine signal involved is not known. There is evidence supporting the induction of IFN-τ gene expression by maternal granulocyte colony-stimulating factor (GM-CSF) and interleukin-3 [24] although effects of GM-CSF have not always been observed [25].

The mechanism by which IFN-τ blocks luteolysis involves disruption of the control of $PGF_{2\alpha}$ synthesis in the uterus. In the cow, IFN-τ induces synthesis of linoleic acid which, in turn, acts as a competitive inhibitor to cyclooxygenase 2 (COX-2) that is the rate-limiting enzyme for $PGF_{2\alpha}$ synthesis [26]. In addition, IFN-τ acts through a transcriptional-dependent mechanism to decrease the stability of COX-2 mRNA [27]. Induction of $PGF_{2\alpha}$ synthesis by endometrial epithelial cells requires actions of both estradiol and oxytocin. In sheep, IFN-τ reduces estrogen receptor-α gene expression, which in turn leads to reduction in amounts of oxytocin receptor [10].

The degree to which immunoregulatory functions of IFN-τ are important for pregnancy is unclear. In fact, the endometrium appears resistant to some of the actions of IFN-τ. Thus, although like other interferons, IFN-τ inhibits lymphocyte proliferation and causes activation of NK cells [17, 18], there was no noticeable effect of the conceptus on endometrial lymphocyte populations at day 16 of pregnancy in cattle [28]. A possible amplifying effect of IFN-τ on presentation of placental antigens through upregulation of class I MHC molecules is limited by the fact that uterine luminal epithelial cells (although not stroma or glandular epithelium) are resistant to this action of IFN-τ [29]. Similarly, endometrial epithelium and stroma are resistant to the antiproliferative action of IFN-τ [30]. There are a large number of endometrial genes whose expression is changed by IFN-τ, however, and some of these could conceivably alter the immune status of the conceptus-maternal interaction. Among these are monocyte chemotactic protein-1 and 2 in endometrial eosinophils [31] and GM-CSF in the luminal epithelium [32].

The Uterine Serpins

The uterine serpins are members of a large superfamily of proteins that, prototypically, fold into a conserved structure and inhibit serine proteinases through a unique suicide-like mechanism [33, 34]. Uterine serpins have been identified in sheep [35], cattle [36], goats [37], and pigs [38] and are therefore more widely distributed phylogenetically than the IFN-τ genes. It has been estimated that uterine serpins diverged at some point before the divergence of mammals [39] and it is possible that uterine serpins are widely distributed among mammalian orders. Depending on analytical methods used, the uterine serpins are classified either as a distinct serpin clade or as a highly diverged group of the α_1-antitrypsin clade [33, 39].

Many serpins have evolved to have functions distinct from proteinase inhibition and this appears true for uterine serpins. The best studied of these, the ovine uterine serpin (OvUS), can inhibit proliferation of αβ T lymphocytes [11] and has been proposed to protect the conceptus from destruction by the maternal immune system [40]. The porcine uterine serpins form complexes with the iron-containing protein, uteroferrin [41], and may be involved in metabolism of that protein. In addition, OvUS exhibits weak pepsin inhibitory activity [36, 42] and can bind to IgA and IgM [43] and activin [44].

All uterine serpins are products of endometrial epithelium [predominately glandular epithelium for most of pregnancy; see 45, 46]. The major signal for induction of synthesis for the ovine and bovine proteins (and presumably caprine

and porcine proteins) is progesterone [47] although placental lactogen or growth hormone can also increase OvUS mRNA in ovariectomized ewes treated with progesterone and IFN-τ [48].

The diversity of effects of OvUS on cells of the immune system and its potential mechanism of action have been reviewed [11]. This protein can inhibit activation of T cells induced by a variety of stimuli, NK cell activation in culture, NK cell-mediated abortion in vivo, and antibody production in response to T cell-dependent antigen, ovalbumin. The protein appears not to inhibit γδ T cells and the resistance of these cells to OvUS may explain in part why their numbers in the luminal epithelium of the endometrium rise in mid and late pregnancy [8]. What is unusual about OvUS as an immunoregulator is that it is not very potent – concentrations in the 100–500 μg/ml range are required to inhibit T cells and NK cells in vitro. These concentrations are well within the physiological ranges of OvUS in uterine fluid, however, and the inhibitory effects of OvUS are likely to be physiologically relevant. Nonetheless, the fact that the protein is only active at high concentrations suggests that it acts to inhibit lymphocytes through some unusual mechanism. Perhaps, the protein has a low affinity for an immunosuppressive receptor or competes with the native ligand for receptors required for lymphocyte activation.

Relevance to Other Species

Whether or not IFN-τ or uterine serpins play an important role in species other than the ones mentioned in this review depends upon their phylogeny. IFN-τ did not arise until soon before pecoran ruminants emerged [12] and therefore one would not find IFN-τ genes in orders of mammals that diverged before this time. Conversely, the early emergence of uterine serpins may mean that these proteins are produced in the uterus of a wide range of mammalian species. It may, however, be that the uterine serpin genes were not retained as functional genes in all species or exhibit expression in tissues other than the uterus.

Regardless of their role in species other than ruminants, there is clinical value in using the ruminants as a source of gene products with therapeutic potential. IFN-τ is less cytotoxic than other type I interferons [15] and has been used to treat experimental allergic encephalomyelitis in mice [49]. IFN-τ also inhibits human immunodeficiency virus replication [50]. Ovine uterine serpin is also active against murine [51, 52] and human cells [53] and either the uterine serpins or a more potent derivative of the serpins may prove useful in regulating lymphocyte proliferative responses.

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Intravenous Immunoglobulin in the Prevention of Recurrent Miscarriage: Does It Work?

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Abstract

Immunological disturbances play a role in the majority of patients with recurrent miscarriage (RM) and therefore treatment with intravenous immunoglobulin (IvIg) has been tested in patients with RM in several trials. Seven placebo-controlled trials that were extremely heterogeneous with respect to patient characteristics and treatment procedures were carried out. One trial found that IvIg significantly improved pregnancy outcome in all patients whereas the remaining trials could either detect no treatment effect at all or only an effect in subsets of patients. In a meta-analysis, the pooled odds ratio for a new live birth in IvIg- versus placebo-treated patients with RM after a birth (secondary RM) was 1.60 (95% CI = 0.70–3.66). IvIg seems to be efficacious in patients with repeated second trimester intrauterine fetal deaths since it significantly ($p < 0.01$) increased the live birth rate in this subset compared with placebo. In most trials the design was suboptimal with regard to detecting any treatment effect of IvIg in RM due to low doses or starting the treatment late. A new large placebo-controlled trial should be conducted in RM patients with secondary RM or repeated second trimester fetal deaths and sufficient IvIg doses should be given with optimal timing.

Introduction

Recurrent miscarriage (RM) defined as three or more consecutive miscarriages affects 0.5–1% of all women. In a minority of the couples causes such as parental chromosome abnormalities or significant uterine malformations can be found and some cases are probably the result of repeated de novo fetal chromosome aberrations in pregnancies of karyotypically normal parents [1]. In the remaining cases one or more immunological or coagulation abnormalities can

often be identified although none of these factors has been documented on its own to be sufficient to cause miscarriage.

Knowing that immunological disease is often caused by cellular autoimmune reactions exclusively localized in the target organ, a demand for the demonstration of immunological disturbances in peripheral blood is not necessary to propose an immunological etiology for an RM case. If anatomical, chromosomal, endocrinological or coagulation disorders are excluded it may be assumed that the etiology is mainly immunological.

Three approaches have been tested as treatments of RM suggested to be caused by immune disturbances: prednisone, active immunization with allogeneic lymphocytes from the partner or third party donors and intravenous immunoglobulin (IvIg). Prednisone seems to be more harmful than beneficial in RM patients positive for various autoantibodies [2]. The use of allogeneic lymphocyte immunization using partner lymphocytes or donor leukocytes was in meta-analyses found to be associated with odds ratios (OR) for life birth of 1.05 and 1.39, respectively, compared with placebo [3], neither of which was significantly elevated relative to 1. However, lymphocyte immunization may be efficient in the subset of women with primary RM who are negative for autoantibodies or lymphocyte antibodies [4].

IvIg exhibits a documented effect in many immunological disorders. The mode of action is probably multiple [5] but some documented effects are modulation of cytokine production, blockage of lymphocyte receptors, inhibition of autoantibody formation, neutralization of activated complement components and induction of apoptosis of activated lymphocytes.

Studies of IvIg Treatment of RM

In RM patients, a number of uncontrolled trials [6, 7] of IvIg treatment have been carried out with apparently favorable results. However, without an untreated control group it is impossible to evaluate the effect since the spontaneous prognosis in most RM patients is good.

Seven placebo-controlled trials of IvIg treatment including 343 RM patients [8–14] have been published so far. The results have been very different with one trial showing a significant treatment effect [10], another showing a strong trend towards a treatment effect [9] whereas the others did not demonstrate any beneficial effect at all. Table 1 shows that the trials were very different with regard to the number of previous miscarriages suffered by the patients, the number of patients with RM after a birth (secondary RM) and frequency of patients with autoantibodies. In several trials no relevant information was given. Table 2 shows data about the treatment protocols used. There was an

Table 1. Relevant clinical and paraclinical background variables in seven published placebo-controlled trials of IvIg treatment for RM

	Studies (Ref. No.)						
Proportion with characteristics	[8]	[9]	[10]	[11]	[12]	[13]	[14]
3 or more losses	100	100	<100	<100	100	100	100
4 or more losses	19	74	?	53	21	22	100
Secondary RM	0	79	~50	51	0	51	53
Autoantibody positive	>0	27	0	0	0	0	43

Values represent the percentage.

extreme diversity between the trials with regard to the starting time of the first infusion, the number of infusions given and the amount of IvIg/placebo given at each infusion.

In 1999 a meta-analysis of the six placebo-controlled trials published at that time was carried out [15]. The overall live birth rates for IvIg and placebo were 60.8 and 59.1%, respectively (OR = 1.14, 95% CI 0.66–1.95, p = 0.7). However, in the secondary RM group, the respective live birth rates were 57.5 and 51.7% (OR = 1.26, 95% CI = 0.48–3.30) pointing towards a better effect in secondary than in primary RM. In 2002, our group published a new placebo-controlled trial of IvIg in RM [14]. In this trial no overall effect of IvIg could be found; however, among the 25 patients with secondary RM the live birth rate was 50% in the IvIg group compared with only 23% in the placebo group. When these results are combined with the results from the previous meta-analysis, the OR for live birth in IvIg-treated secondary RM patients is 1.60 (95% CI 0.70–3.66; p = 0.27) (fig. 1).

Patient Subsets and Treatment Protocols May Determine Outcome of IvIg Studies

It is clear that so far there is no scientific evidence that IvIg should be offered to RM patients in general [14, 15]. However, tables 1 and 2 clearly show that the patient populations included in the placebo-controlled trials and the treatment protocols used were extremely heterogeneous and it is questionable how much information is obtained by the combination of all patients from these trials in a meta-analysis. Knowledge about patient subsets and treatment modalities being

Table 2. Relevant details of the treatment protocols in seven placebo-controlled trials of IvIg treatment for RM

	Studies (Ref. No.)						
	[8]	[9]	[10]	[11]	[12]	[13]	[14]
IvIg dose/ infusion[1], g	20–30	20–40	30	30	25	20	40–75
Placebo	5% alb.	5% alb.	0.5% alb.	sal.	5% alb.	sal.	5% alb.
Time of 1st infusion	5th to 8th week	5th week	b.c.	b.c.	5th to 7th week	6th to 9th week	5th week
Infusion interval[2], weeks	3	1	4	?	3	3	1

alb. = Albumin; sal. = saline; b.c. = start of infusions before conception.

[1]In case of a range indicated, the first dose was greater than subsequent doses or the doses were dependent on body weight.

[2]In the first trimester.

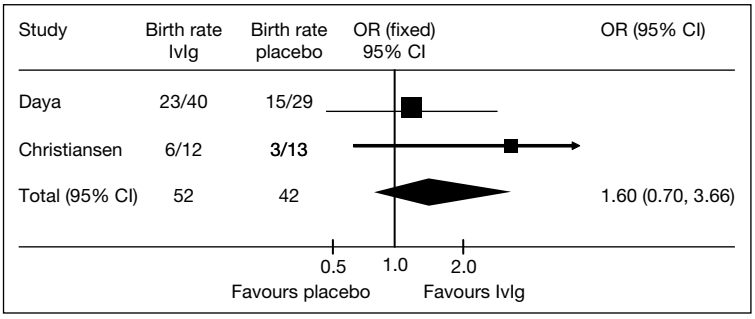

Fig. 1. Combined OR for live birth in patients with secondary RM treated with IvIg or placebo using data from Christiansen et al. [14] and Daya et al. [15].

associated with the largest treatment effect is necessary for the optimal design of future placebo-controlled trials of IvIg treatment and it is worthwhile to take an extensive look at these issues.

Patient Subsets

Four patient subsets should be focused on in future randomized trials because data from trials conducted so far or theoretical considerations indicate that these may benefit mostly from IvIg therapy. The four subsets are (1) secondary RM

Table 3. Live birth rates in RM patients with a history of at least two second trimester intrauterine fetal deaths and a total of at least three miscarriages

	IvIg	Placebo	OR (95% CI)	p
Live birth	7 (88%)	1 (11%)	56.00 (2.93–∞)	<0.01
Miscarriage	1	8		

Results from a pilot study and two placebo-controlled trials. 95% CI = 95% confidence interval.

patients, (2) patients with repeated second trimester fetal deaths, (3) patients with multiple (≥4) miscarriages and (4) patients with autoantibodies.

(1) Secondary RM is defined as RM after a pregnancy that progressed beyond the 28th or 20th gestational week [16]. There are some interesting features of secondary RM: allogeneic lymphocyte immunization seems to display a much smaller therapeutic effect in patients with secondary RM as compared with those with primary RM [17] and women with secondary RM are more often positive for lymphocytotoxic or blocking antibodies than patients with primary RM [16], indicating that the immune system of the former group has been challenged more heavily by paternal/fetal antigens during previous pregnancies [16]. Recent results [18] also point towards a role for immunization against male specific minor histocompatibility (HY) antigens in the etiology of secondary RM. There are thus many indications that secondary RM represents a separate immunological entity and this stresses the necessity for testing IvIg separately in this subgroup. The results from placebo-controlled trials conducted so far (fig. 1), showing that among patients with secondary RM, IvIg treatment most likely increases the chance of a live birth 1.60 times compared with no treatment, are in accordance with this hypothesis.

(2) Repeated deaths of morphologically and genetically normal fetuses in the uterus between the 14th and 28th gestational week occur quite rarely but are very distressing. Only in our two placebo-controlled IvIg trials [9, 14] and our pilot study [19] was information provided about the inclusion of patients with repeated second trimester miscarriages. In table 3 the pregnancy outcomes in the three studies of this patient subset are summarized; all these patients were treated before 2000. It may indeed turn out to be the main target group for IvIg therapy due to the apparently very large therapeutic benefit: absolute improvement of live birth rate = 77% and OR for live birth 56.0 (95% CI 2.93 to ∞, $p < 0.01$). From 2000 until now we have continued to offer IvIg to patients with repeated second trimester intrauterine fetal deaths at our clinic [20] and the live birth rate is still excellent (89%) in this group of women who without treatment only have an 11% chance of live birth (table 3).

(3) The number of previous miscarriages is generally acknowledged as the most important predictor of subsequent pregnancy outcome in women with RM [17]. Furthermore, there is plenty of support for the hypothesis that the chance of an immunological etiology for RM increases with an increased number of previous miscarriages: the prevalence of particular HLA-DR alleles or low serum concentrations of mannan-binding lectin [21] and of particular HLA-G alleles [22] increases with the number of previous miscarriages. The chance of other etiologies leading to miscarriage such as fetal chromosome abnormality also decreases with the number of previous miscarriages [23]. In theory we would thus expect a larger treatment effect of IvIg in patients with multiple miscarriages than among those with fewer miscarriages and this is supported by the results of an uncontrolled trial [24]. However, in none of the placebo-controlled trials were data available which allowed an estimation of the treatment effect in the patients according to the number of previous miscarriages. In conclusion, although theoretical considerations suggest that IvIg may be more beneficial among patients with multiple compared with fewer miscarriages we still lack evidence from treatment trials to support this.

(4) In theory, RM patients with autoantibodies should be a target group for IvIg therapy. IvIg is obviously only expected to be beneficial in patients who miscarry due to immunological disturbances. No immunological test has a documented high specificity to identify women who miscarry repeatedly due to immunological disturbances but there is some consensus that the presence of autoantibodies and especially antiphospholipid antibodies may identify such patients. Most placebo-controlled trials of IvIg in RM have excluded patients with autoantibodies and only in our two trials was it possible to extract data concerning pregnancy outcome in autoantibody-positive patients. During the first trimester IvIg treatment did not suppress autoantibody titers [9] and the live birth rates were similar or lower in IvIg-treated compared with placebo-treated anticardiolipin-positive patients [14]: 36 versus 45%, respectively. There is thus no documentation that IvIg has a particular high effect on autoantibody-positive RM patients.

Treatment Protocols

As shown in table 2, the protocols used in the placebo-controlled IvIg trials were very different emphasizing the fact that nobody knows which treatment schedule is optimal. By analyzing success rates in the different trials according to (1) the IvIg doses used and (2) the starting time of the infusions much can be learned about which protocols may be most interesting for future testing.

(1) Since the mode of action of IvIg in most immunological disorders is not fully clarified, the doses used in these disorders are empirical and often lack consensus. Frequently used regimens are 0.4 g/kg body weight daily for 5 consecutive

days for idiopathic thrombocytopenic purpura [25] or 1 g/kg body weight/day for 2 days at 4 weeks' intervals for severe asthma [26]. The doses used in most RM trials have generally been much smaller than those used in other disorders and only in our trials [9, 14] have doses been used which approach the doses mentioned earlier, resulting in the mean serum IgG concentration increasing to 23.3 g/l from 11.4 g/l before the first infusion [14]. In contrast, the doses used in the other trials are not expected to result in any significant increase of serum IgG concentrations.

(2) The starting time of IvIg infusions may be crucial since obviously any therapy for RM should start before the embryo is dying or already dead. The majority of miscarriages in RM patients occur before week 8 and in these cases the embryo very often exhibits signs of impaired growth from week 6 as measured by ultrasound or hormones. It is therefore important to start IvIg infusions from gestational week 5 or before if a beneficial effect is to be obtained. In spite of this, in three trials [8, 12, 13] infusions were only started in most of the patients in week 6–8 after the detection of fetal heart action. Pregnancies being viable at this relatively late time of gestation display a fair spontaneous prognosis for being successful because a considerable part of the 'at risk' time has passed at inclusion [27]. The success rate in the placebo group is thus expected to be high, which is also evident in the relevant trials [8, 12, 13] where success rates in the placebo group of 68–79% were detected; with such good spontaneous outcomes it is impossible to detect any therapeutic effect of IvIg. Starting IvIg in gestational week 5 may even be too late for many pregnancies. There are indications that it takes weeks to obtain some of the immunomodulating effects of IvIg such as downregulation of B lymphocyte clones and selection of T lymphocyte repertoires. It is therefore reasonable to believe that starting infusions some time before embryonic implantation might improve the effect. In two trials [10, 11] this approach was indeed used and one of them reported a significant treatment effect [10], indicating that preimplantation IvIg infusion is a modality which should be further tested. In a meta-analysis there were some indications that the start of IvIg before conception is beneficial in women with primary RM [15].

Negative Aspects of IvIg Use in RM

There are two negative aspects of IvIg therapy that must be considered when deciding whether to use it in RM patients or not: (1) the potential harmful effects and (2) the economic costs of the drug.

(1) The harmful effects can be divided into allergy and direct effects on organs and the risk of transmission of infectious agents. A severe immunological reaction against the (small) IgA content in IvIg can develop in IgA-deficient

patients; however, by screening for IgA deficiency (found in approximately 1/800) before IvIg infusion this complication can be avoided. Allergic reactions against other components in IvIg are normally slight (skin rash, arthralgia). Although in our clinic we administer quite high amounts of IvIg to our RM patients [20] we only had had to cancel infusions in one out of more than 250 IvIg-treated patients due to unacceptable side effects (severe skin rash and urticaria).

A series of pathogens poses a confirmed or theoretical risk of transmission by plasma products. Hepatitis A, B and C, HIV-1 and HIV-2, HTLV-I/II and parvovirus 19 can without doubt be transmitted by blood products whereas the prions causing the variant form of Creutzfeldt-Jacob disease (bovine spongiform encephalopathy = BSE) in theory might be transferred by plasma products although this has never been documented. Considerable achievements have been made in the reduction of the possible risk of transmission of infections agents. The main steps in obtaining the current high safety of plasma products are: (i) rigid donor selection procedure, (ii) screening of donations in order to exclude infectious donations and quarantining of batches of plasma for 60 days, and (iii) validated steps for elimination and/or inactivation of potentially infectious agents. Elimination and/or inactivation of pathogens can be undertaken by disrupting the viral envelope by solvent/detergent treatment, pasteurization, or ionic disruption by low pH treatment. Cold-ethanol fractionation methods used in the production of some IvIg brands seem to reduce significantly the concentration and infectivity of prions added to test IvIg products. Nanofiltration has recently been introduced in the manufacturing processes of IvIg and this method is able to remove nonenveloped viruses that are not inactivated by other methods and it can also eliminate prions causing BSE [28]. Overall, using the procedures mentioned has resulted in a high pathogen safety of currently available plasma products. Indeed no transmission of pathogens after IvIg infusion has been reported since the early 1990s [28].

(2) The economic costs of IvIg are unfortunately high for two reasons: the demand for the drug exceeds its supply which is dependent on the number blood donations and the production process; this process which includes screening for and inactivation of pathogens is complex and expensive. In Denmark, the price of IvIg paid by the hospitals to the manufacturers is approximately 48 Euros/g. A patient with secondary RM and at least four first trimester miscarriages will in our clinic receive seven infusions of 25 g IvIg, which cost 8,400 Euros. According to our placebo-controlled trials, the treatment will increase the live birth rate from 23 to 58%. The number of patients needed to treat to achieve one additional live birth can be calculated as 2.8 patients and each live birth will thus cost $2.8 \times 8{,}400 = 23{,}520$ Euros. This amount is only slightly higher than the cost for each live birth after IVF/ICSI treatment provided that 60% of those

starting treatment will end up having a live birth and that they on average will need two treatment cycles to achieve the live birth. With respect to the patients with repeated second trimester losses, the expense of the treatment is higher (13,200 Euros) since we continue treatment for a longer time during pregnancy in these patients but, according to table 3, the number of patients needed to treat to achieve one live birth is only 1.3 and the cost per treatment-related live birth is thus 'only' 17,160 Euros. Knowing the extreme grief and distress caused by second trimester fetal deaths and the high hospital expenses related to repeated ultrasound examinations, hospitalization for several days during the medical induction of abortion of the dead fetus, anesthesia for the surgical evacuation of the uterus and the autopsy and karyotyping of the fetus, 17,160 Euros seem to be a low price to avoid a late fetal death.

Conclusions

The current knowledge about IvIg treatment in RM is primarily based on seven very heterogeneous placebo-controlled trials comprising a total of only 343 patients. There are indications that IvIg has a strong effect on women with repeated second trimester fetal deaths (table 3) and in this subgroup the treatment is probably cost-effective. However, the only documentation for this strong treatment effect comes from the retrospective analysis of the results of previous trials conducted in one clinic and optimally a new prospective placebo-controlled trial focusing on this subgroup should be conducted. It may unfortunately be very difficult to do such a trial due to the low prevalence of repeated late fetal deaths and the reluctance of these extremely distressed patients to participate in a placebo-controlled trial.

Among the patients with secondary RM, the pooled OR for live birth in IvIg-treated versus placebo-infused women was 1.60. A new prospective placebo-controlled trial focusing on patients with secondary RM and using an IvIg infusion protocol associated with a 76% success rate in this subgroup [20] should be conducted and it may still be possible to recruit patients to such a trial. However, the pharmaceutical companies producing IvIg seem to be very reluctant to support further placebo-controlled trials in RM patients because they accept the conclusion from the Cochrane meta-analysis as it stands now: that there is no indication for an effect of IvIg on RM. As discussed in this review, there are indeed a lot of strong indications that IvIg display effects in (large) subsets of RM patients using the right protocols. The first seven placebo-controlled trials have given us enough information to enable us to prepare a protocol which could be tested in a new large placebo-controlled trial and which would have a very good chance to document an effect. It would be disastrous for the progress

of the treatment possibilities of RM if research into this promising treatment is being stopped prematurely due to the Cochrane analysis and its interpretation. If this were the case the analysis would have failed to fulfill one of its main purposes: to help the clinical researchers to prepare and conduct trials, which can give definitive answers to the questions being posed.

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IgE-Mediated Allergy against Human Seminal Plasma

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Abstract

Human seminal plasma hypersensitivity has to be differentiated from allergic reactions to latex, spermicidal agents, local anesthetics or components of lubricants. The present review article discusses IgE-mediated allergic reactions (type I) to specific components of the seminal plasma. Such incidents are rare, even though there seems to be a considerable number of unreported cases. Since the first publication in 1958, human seminal plasma allergy has been increasingly recognized, and approximately 80 cases have been described. Most affected women are younger than 40 years, presenting with an atopic family history. Anaphylaxis to components of the seminal plasma is not always associated with infertility. Complaints occur immediately or within 1 h after contact with seminal plasma. Local reactions include itching, burning, erythema and edema in the vulvar region or other sperm contact sites. Systemic reactions are experienced as dyspnea, dysphagia, rhinoconjunctival complaints, generalized urticaria, angioedema, gastrointestinal symptoms, exacerbation of existing atopic eczema or anaphylactic shock. Recently, it has been reported that human seminal plasma anaphylaxis may also present as 'vulvar vestibulitis syndrome' or 'burning semen syndrome'. These symptoms may occur during the first sexual intercourse. Some results are indicative of allergens originating from the prostate, prostate-specific antigen being clinically relevant. The diagnosis of human seminal plasma allergy is based on history, demonstration of specific IgE antibodies in the serum and skin tests. Therapeutic options include allergen avoidance by use of condoms and attempts at desensitization.

Introduction

Reactions after contact with human ejaculate may have various origins and are not always referable to true sensitization against allergens contained in the

seminal plasma. As symptoms generally occur after sexual intercourse or in the context of sexual activities, they may be difficult to differentiate from allergic reactions to latex (condoms), spermicidal agents (vaginal contraceptives), local anesthetics (in some condoms) or components of lubricants, especially in case of inaccurate history taking and diagnosis. The present review article discusses IgE-mediated (type I) immediate-type allergic reactions to specific components of the seminal plasma.

In the literature there are reports of cellular (type IV)- and immune complex (type III)-mediated allergies as well as immediate-type reactions to antigens contained in the seminal plasma [1–4]. Although such symptoms are generally rare, there seems to be a considerable number of undetected or untreated cases. As early as 25 years ago it was pointed out that the first diagnosis was more frequently made by affected women than by their physicians [5].

An example of nonallergic symptom reaction to ejaculate are uterine contractions caused by prostaglandins in the seminal plasma [6]. Casuistic reports have also described anaphylactic reactions after exposure to ejaculate in women who were highly sensitized to particular allergens (e.g., drugs or food) which had been consumed by their sexual partners and reached the seminal plasma. These were not seminal plasma-specific allergens, but drugs such as thioridazine, penicillin and vinblastine [7–9], food such as walnuts [10] or food components e.g. in Coca-Cola [11].

A special form of hypersensitivity to human ejaculate was reported by Witkin et al. [12]. They discussed the case reports of 3 women with recurrent vaginitis, in whom temporary improvement had been achieved after therapy with antihistamines, but whose symptoms could only be safely relieved after the use of condoms. In contrast to women who are sensitive to seminal plasma-specific allergens, 2 of these 3 patients had only symptoms after sexual intercourse with their current sexual partner, but not with other men. IgE antibodies to components of the partner's seminal plasma or that of control persons were not demonstrated in the vaginal fluid of these women. On the other hand, 2 of the 3 men had IgE antibodies in their seminal plasma that reacted with components of the vaginal fluid of their partners or (in one case) also with that of another woman. The vaginal fluid fractions contained epithelial cells and the microbial resident flora, but were not further characterized. The partner whose IgE antibodies in the seminal plasma did not react with components of the vaginal fluid had significantly higher IgE levels (29 kU/l) than the other two men (9 and 12 kU/l). Witkin et al. [12] hypothesized that IgE from the partner's seminal plasma would bind to mast cells or basophils of the vaginal mucosa and then react with allergens from the vaginal tract or seminal plasma. The symptomatology of vaginitis is explained by the release of histamine and other mediators. The allergens involved have not been further characterized. The authors believe that IgE-mediated reactions to

components of *Candida albicans* are possible, but do not exclude nutritional influences, as the patient reported increased symptoms when her partner had drunk beer prior to sexual intercourse.

IgE-Mediated Reactions to Specific Components of the Seminal Plasma

Frequency

True hypersensitivity to antigens in the seminal plasma is rare, even though there seems to be a high number of unreported cases [13]. To date, about 80 casuistic or serial reports on type 1 reactions to seminal plasma have been published, the first by Specken [14] in 1958, and the first in English by Halpern et al. [15] in 1967. Specific IgE antibodies to seminal plasma are frequently found in the serum of women, especially in cases of high total IgE levels. Vogt [16] reported on positive radioallergosorbent test (RAST) results with demonstration of IgE antibodies to seminal plasma in 31.8% of 44 women with total IgE levels above 1,000 kU/l. However, it has to be considered that false-positive RAST results may occur with such high IgE values. In the studies mentioned, this is supported by the observation that none of the women complained about anaphylactic reactions after exposure to seminal plasma.

Within a targeted inquiry among 1,073 women who themselves assumed that they were hypersensitive to ejaculate, 130 women reported localized ($n = 46$) or generalized ($n = 84$) symptoms occurring after exposure to seminal plasma, which could be prevented by the use of condoms [17].

Symptomatology

The clinical symptoms of seminal plasma anaphylaxis comprise a wide spectrum ranging from local reactions to anaphylactic shock. Subjective and objective complaints occur immediately or within 1 h after exposure to ejaculate, rarely thereafter. Systemic reactions were noted immediately, while local reactions generally occurred with a delay in time [18]. Local reactions include itching, burning, erythema and edema in the vulvar region (vulvovaginitis) or other sites of ejaculate contact. In addition, pelvic pain has been reported [15]. Bernstein et al. [19] described a 'burning semen syndrome' in Gulf War couples. Babula et al. [20] found specific IgE antibodies against seminal plasma in 30.8% of 52 women with vulvar vestibulitis syndrome.

A special reaction to seminal plasma was observed by Best et al. [2] in a woman who developed recurrent local skin changes within 2–48 h after sexual intercourse, which were clinically similar to fixed drug reactions.

Systemic hypersensitivity reactions to components of seminal plasma include dyspnea, asthma, dysphagia, rhinoconjunctival complaints, generalized urticaria, angioedema, gastrointestinal symptoms with vomiting and diarrhea, exacerbation of existing atopic eczema and anaphylactic shock.

Discussing the case reports of 32 patients with hypersensitivity to their partner's ejaculate, Presti and Druce [18] found anaphylactic reactions and dyspnea each in 22%, while itching, local pain and edema occurred most frequently with 47–84%. Symptoms were reported to occur with gradual worsening after several instances of sexual intercourse and with a reduction in the time of onset [13].

Age of the Patients

The majority of women with allergic reactions to components of the seminal plasma are between 20 and 30 years old. While Presti and Druce [18] listed only 1 patient under 20 years, our own clinical observations indicate that with increasing sexual contacts in younger years, a higher number of younger women complain of such symptoms. Women over 40 years are less frequently affected.

Predisposing Factors

The high number of women complaining about symptoms of hypersensitivity to seminal plasma after their first sexual intercourse is striking. Presti and Druce [18] observed such statements in about 41% of their patients. This may be traceable to the fact that seminal plasma antigens cross-react with other allergens that are still unknown. A further explanation would be that the women had been sensitized by nonintravaginal sperm exposure prior to their first sexual intercourse.

More than 50% of the affected women have other allergic symptoms such as allergic rhinoconjunctivitis or a positive family history of atopic diseases (hay fever, atopic eczema, bronchial asthma) [18]. Major studies did not reveal an association with atopic diathesis, but a relation to food allergy [17]. Familial disposition concerning allergic reactions to components of the seminal plasma has been reported only once in 4 women of a family [21].

In some cases, first symptoms were preceded by pregnancy, gynecological interventions such as hysterectomy, sterilization, intrauterine device insertion or urological operations (prostatectomy) of the partner [22–27]. More than 30% of women with vulvar vestibulitis syndrome and specific IgE antibodies against seminal plasma report symptom initiation after a yeast infection [20].

The causes of altered reactivity of the female immune system towards antigens of the seminal plasma have been speculative. Apart from the aforementioned

cross-reacting antigens, altered immunosuppressive effects of the seminal plasma, impaired immune defense in the female genital tract and similar HLA antigens of both partners have been considered [18].

Allergens in Seminal Plasma

In the majority of cases, hypersensitivity to seminal plasma is not confined to a particular male sexual partner [28]. Thus, the allergen is not specific to an individual. However, Frankland and Parish [5] reported a woman who had had sexual intercourse and 3 children with her previous husband without any symptoms of hypersensitivity, but experienced generalized intolerance reactions to the seminal plasma of her second husband. As the authors did not perform any skin tests with the seminal plasma of other men, it cannot be excluded that the woman's sensitization occurred purely by chance after sexual contact with her second husband.

However, another casuistic report describes an intolerance reaction exclusively to the ejaculate of the sexual partner [29]. After intercourse, the 21-year-old woman experienced vulvar itching and edema as well as generalized exanthema. During the following allergological testing, scratch tests with her partner's seminal plasma revealed urticarial reactions on her upper arm. Repeated tests with the seminal plasma of another man did not cause any skin reactions, nor were any reactions observed when her partner's seminal plasma was used for skin tests in a female volunteer. There are also reports with experience of allergic reactions to seminal plasma of four or more different partners [24].

A typical example of reaginic-type sensitization to seminal plasma is the case described by Halpern et al. [15] of a 29-year-old woman who developed all the above-mentioned symptoms 15–30 min after sexual intercourse with her husband. In contrast to several dilutions of the husband's seminal plasma, scratch tests with the seminal plasma of bulls, horses, rabbits and guinea pigs did not cause positive reactions, and washed spermatozoa of the partner were well tolerated on the skin. The allergen was apparently not only contained in her partner's seminal plasma, as the patient also reacted to that of healthy donors. Diluted seminal plasma specimens from her partner were well tolerated by female volunteers. In a sensitized patient, Levine et al. [30] performed scratch and intracutaneous tests with the seminal plasma of 15 men. Local urticarial reactions occurred with all specimens. Thus, the allergen(s) seem to be species-specific and not individually bound.

Positive skin reactions are generally only observed with seminal plasma, but not with the partner's serum or saliva [5, 15]. However, Freeman [26] reported on a woman who developed positive skin reactions to both her partner's seminal plasma and sweat. Another casuistic report indicates a temporal association between ejaculation and antigen occurrence in the serum of the

male sexual partner, resulting in increased histamine release from leukocytes of the affected patient [31].

By chromatographic investigations the molecular mass of allergens that are responsible for allergic reactions to seminal plasma was defined to range between 12 and 75 kDa [18, 32]. After isoelectric focusing and subsequent skin testing, allergenic fractions were found between pH 5.4 and 6.6 [32], pH 6.6 and 7.5 [33] as well as pH 8.4 and 8.6 [34]. In a study by Levine et al. [30], 90% of the allergenic activity of components of the seminal plasma was found in a fraction with a molecular mass between 20 and 30 kDa. Thus it appears that it is not only one allergen that causes the symptoms in women.

The site of origin of the allergen(s) may be the prostate. Positive reactions were reported after testing with expressed prostatic secretions and prostatic tissue extractions on the skin [29, 35]. Scratch and intracutaneous tests were also positive when seminal plasma of vasectomized men had been used [30]. This is a further indication that the allergens cannot derive from the testes or epididymis. Park et al. [36] found nine allergens with molecular masses of 18, 20, 33, 38, 40, 50, 65, 75 and 100 kDa in the seminal plasma of a man whose wife reacted with vulvar edema, generalized urticaria, facial swelling and dyspnea after exposure to seminal plasma.

In summary, there is no coherent information concerning the precise characterization and origin of allergens responsible for immediate-type allergy to seminal plasma. Some results indicate that allergens derive from the prostate, prostate-specific antigen thus being clinically relevant. Major series (e.g. prick tests with seminal plasma), however, are not justified because of potential risks of infection (e.g. HIV).

Diagnosis

Careful history taking is an essential part of the diagnosis. Thus, potential indications of sensitization to other antigens that are not contained in the seminal plasma (latex in condoms, vaginal contraceptives, lubricants) may be clarified beforehand. Particular attention should be paid to the course of symptoms after exposure to semen (beginning and duration of complaints). The patient's symptoms have to be evaluated by careful questioning. It is important to differentiate between local and generalized allergic reactions. If potentially life-threatening symptoms are reported (decrease in blood pressure, dyspnea), the prescription of an 'emergency kit' should already be considered at first consultation (see therapy).

As described in the section 'Predisposing Factors', the affected women's own history and family history often provide a clue to atopic diseases that

should be specifically asked for during the first consultation. Shah et al. [37] reported an association between episodic asthma in young, married female patients and seminal plasma allergy.

Apart from the patient's history, diagnosis of allergy is of utmost importance to identify hypersensitivity to seminal plasma. In this context, the discussion is limited to the routine diagnostic setting.

In the in vitro diagnosis it is useful to analyze the patient's serum for specific IgE antibodies against seminal plasma. The relevant antigen for the RAST is commercially available. In addition, it is recommended to determine the total serum IgE levels. The search for specific antibodies to seminal plasma is not always successful. In their review of 32 women, Presti and Druce [18] reported 6 positive, 12 negative and 14 indeterminate RAST results. By means of enzyme-linked immunosorbent assay (ELISA) it was possible to demonstrate IgG and IgA in addition to IgE antibodies to seminal plasma [38]. Corresponding assays are commercially available today.

More time-consuming, though used in many allergy labs, are assays for detection of allergen-induced histamine release from peripheral leukocytes. Positive results are obtained in some of the patients [18]. A new approach to invitro diagnosis of seminal plasma allergy is offered by the cellular antigen stimulation test-ELISA, which allows analogous measurement of allergen-induced sulfidoleukotriene release.

For scientific purposes, the antigens or fractions can be separated electrophoretically or chromatographically and are then further identified by IgE immunoblotting [36].

Prick and intracutaneous tests are most important in the diagnosis of seminal plasma allergy. Depending on the extent of sensitization, emergency medical services may be required for skin testing. Spermatozoa and seminal plasma are separated beforehand by centrifugation. The test is generally performed with the sperm pellet. However, reactions can only be expected to occur with seminal plasma fractions.

For testing, seminal plasma has been used without previous separation or after fractioning (chromatography, electrophoresis, filtration) at dilutions between 1 and 10^{-8} [31, 38]. Extensive tests using ejaculates of healthy donors or different animal species, which were performed more than 30 years ago, are no longer justifiable. Even if the partner's ejaculate is used, serological and microbiological examinations are necessary to minimize the potential risk of infection (HIV, hepatitis, syphilis). Documented information of the patient prior to testing is recommended.

Skin tests are positive in the majority of cases. In their review of 32 women with seminal plasma allergy, Presti and Druce [18] reported 27 positive, 3 negative and 2 indeterminate results.

The diagnosis should also exclude other diseases with similar symptomatology, e.g. contact dermatitis in the genital region, candida infections or bacterial vulvovaginitis.

Therapeutic Options

Since it is known that the allergen is not an individual allergen, the recommendation by Berger [29] 'that she avoids this gentleman' is no longer valid. Taken prior to sexual intercourse, antihistaminics may relieve symptoms in some patients [21]. If the patient's history indicates that she is highly sensitive and develops generalized and threatening reactions, an 'emergency kit' should be prescribed already at first consultation, similar to that used for bee and wasp venom allergy [39]. In addition to an antihistaminic the set should contain a corticosteroid and possibly an adrenaline aerosol spray. The patient should be instructed about its application in case of unintentional exposure to seminal plasma.

The second immediate recommendation is prevention of exposure. Condoms should be used consistently during sexual intercourse. Kroon [24] reported regression of symptoms and negative prick tests in 3 of 4 women after the use of condoms for 6–12 months. Simultaneous sensitization to both seminal plasma and latex may occur in rare cases [4, 40], for which latex-free condoms are recommended.

Apart from exposure prevention, there have been various attempts at desensitization. The first report was published in 1967 by Halpern et al. [15] whose patient was given injections of undiluted seminal plasma for 2 years, but without success. In contrast, Blair and Parish [41] reported a successfully desensitized patient who had received injections of undiluted seminal plasma at increasing concentrations twice weekly for 6 months.

In a 24-year-old woman with anaphylactic reactions after exposure to seminal plasma, Mittman et al. [42] induced rapid desensitization (2 days) using chromatographically separated seminal plasma fractions. This was followed by subcutaneous injections of allergologically relevant fractions 3 times weekly for 4 months. Thereafter, sexual intercourse was possible without any symptoms, and the couple was asked to have unprotected intercourse every 2–3 days to maintain immunity. No symptoms occurred during the subsequent 6 months. Similar good results of desensitization with fractions of seminal plasma were reported by other groups [32, 38].

A different kind of hyposensitization was chosen by Park et al. [36]. The vaginal mucosa was exposed to 2 ml of 10^{-5} diluted seminal plasma of the husband with 10-fold increments in concentration to undiluted semen at 45-min intervals. Thereafter, the couple had sexual intercourse at intervals of 2–3 days

without any symptoms. Prolongation of the interval to 5 days resulted in slight local itching and vular swelling. Spontaneous pregnancy occurred after 6 months. Clinically successful intravaginal desensitization had previously been achieved by Matloff [43].

Human Seminal Plasma Allergy and Infertility

Human seminal plasma allergy is not always associated with immunological infertility of the patients. However, women who are highly sensitive to components of the seminal plasma cannot have unprotected intercourse so that natural conception is virtually impossible.

In a review article, Presti and Druce [18] reported that 8 of 32 patients with seminal plasma allergy had full-term pregnancies. It cannot be excluded that these occurred prior to sensitization. In 3 of 5 women who had children, Vogt [16] demonstrated specific IgE antibodies to seminal plasma prior to pregnancy. However, all these women were clinically asymptomatic.

If symptoms make unprotected intercourse impossible in couples who want to have children, pregnancy may be achieved after intrauterine insemination with washed spermatozoa [44]. Subcutaneous or intravaginal hyposensitization in the affected women may be an alternative method [25, 36, 45].

Practical Suggestions

In cases of suspected seminal plasma allergy, careful history taking may reveal sensitization against other antigens that are not contained in the seminal plasma (e.g., latex in condoms, vaginal contraceptives, lubricants). Of special importance are local or generalized allergic reactions. If potentially life-threatening symptoms are reported (decrease in blood pressure, dyspnea), the prescription of an 'emergency kit' should already be considered at first consultation. Primarily, the couple should be advised to avoid exposure by using condoms. Thereafter, a specialized center for allergology should be involved.

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Antiphospholipid Antibodies and Reproductive Failure

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Abstract

Antiphospholipid antibodies (aPLs) are characterized as heterogeneous and nonspecific autoantibodies directed against cardiolipin, ph-serine, ph-inositol, ph-acid, ph-glycerol, ph-choline, annexin V, and co-actor β2-glycoprotein I. aPLs occur during various autoimmune diseases, infectious diseases, neurological and kidney diseases, transplant loss, metabolic diseases, and drug abuse. They are also found in connection with reproductive failure. Antiphospholipid syndrome (primary or secondary) has to be treated according to the type and levels of aPLs as well as clinical symptoms (such as repeated pregnancy loss, preeclampsia, repeated missed abortions, unexplained hypertension, repeated delivery of hypotrophic fetuses) by a team of clinicians such as rheumatologists, reproductive immunologists, hematologists, and obstetricians. Based on clinical experience a low dose of heparin/fraxiparine or a low dose of aspirin and corticosteroids is used. This chapter contains up-to-date information about the clinical and laboratory significance of the antiphospholipid syndrome.

Introduction

Antiphospholipid antibodies (aPLs) represent a family of heterogeneous autoantibodies with apparent specificity for negatively charged phospholipids. aPLs are very often detected by enzyme-linked immunoassay using phospholipids [cardiolipin (CL), phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidic acid, phosphatidylglycerol, lupus anticoagulant (LA)], and β2-glycoprotein I (β2-GPI) as a cofactor C. LA is defined as an immunoglobulin, either IgG, IgM or both, which interferes with one or more of the phospholipid-dependent tests of in vitro coagulation. aPLs are classified according to the molecule. aPL isotypes are very often described

as IgG, IgA, IgM; some published papers [e.g. 1, 8, 12, 17] make a connection between the types of aPLs and their associated pathologies.

A Brief Historical Background

The discovery of aPLs began in 1907 when Wasserman [1] introduced a diagnostic test for syphilis, using a saline extract of liver from fetuses with congenital syphilis as the antigen to detect a serum antibody of patients with syphilis. Later on, in 1941, Pangborn [2] isolated serologically active phospholipids from beef heart; she called it CL, and after that all tests detected antibodies to extracted CL. There are a lot of publications [e.g. 3, 9] describing serological positivity to CL without clinical or epidemiological evidence of syphilis. At first, it was found in patients with systemic lupus erythematosus (SLE), where their plasma also demonstrated a unique inhibitor of in vitro coagulation, LA, an immunoglobulin directed against the phospholipid portion of the prothrombin activator complex [e.g. 4, 5]. LA were mostly the domain of hematologists prior to 1983; the development of the aCL antibody assay resulted in great interest in the clinical syndromes associated with their presence [e.g. 6–8].

The term of aPLs has been used in more than 5,300 publications describing not only the problem of reproductive failure. aPLs identification is connected with various autoimmune diseases, obviously with SLE and other autoimmune diseases such as rheumatoid arthritis, systemic sclerosis, Sjögren's syndrome, and others [e.g. 9–11], with infectious diseases (viral, bacterial, spirochaetal, parasitic) such as tropical diseases, tuberculosis, pneumonia, measles, chickenpox, hepatitis, smallpox, parvovirus, rubella, mumps, ornithosis, adenovirus, Lyme's disease, HIV [e.g. 12, 13], in patients with myocardial infarction, pulmonal hypertension, cerebrovascular stroke, and in some kinds of nephropathies [e.g 14]. The catastrophic antiphospholipid antibody syndrome (APA sy) was first defined in 1992 [15] with 'triggering' factors being trauma, anticoagulation disorder, carcinoma, and also infections, much more the viral than the bacterial ones. Today, the presence of aPLs is considered to be a leading risk factor for a patient with thrombotic disease. aPLs have also been identified in some patients with a variety of neurological problems such as transverse myelopathy, chorea, Guillain-Barré syndrome, migraine, and epilepsy, and in some patients untreated recurrent cerebral thrombosis has led to multi-infarct dementia [e.g. 16, 17], or Tourette's syndrome, a familial neuropsychiatric disease (chronic motor and vocal tics) [18]. aPLs have been found in association with the administration of various drugs such as hydralazine, procainamide, valproate, amoxicillin, quinidine, streptomycin, propranolol, phenytoin, suggesting an etiological association [e.g. 19]. aPLs were also studied in sera of alcoholics [20], where antibodies against ph-ethanolamine

were identified significantly more often than in the control group. The same results were also obtained in qualitative and quantitative studies of autoantibodies to phospholipids in diabetes mellitus [21]. Deep metabolic changes in both diseases might result from increased aPLs on the cell plasma membrane (e.g. the change of annexin V and ph-serine localization on trophoblast).

McIntyre's laboratory was the first [22, 23] to report that aPLs (especially antibody against ph-ethanolamine) were associated with hyperacute allograft loss in a patient whose negative pretransplant cross-match transplanted kidney failed to function within hours after transplantation. The same authors [23] reviewed the evidence of aPL levels in association with an increased risk of thrombosis during or after transplantation of kidney, liver, heart, ventricular assist devices, vascular bypass, and bone marrow transplantation.

Laboratory aPLs Examination

Today, ELISA-based immunoassays are only used for detection of antibodies against CL, ph-serine, ph-inositol, ph-glycerol, ph-choline, ph-acid, ph-ethanolamine and β2-GPI. Commercial tests often standardize, which is not satisfactory; the use of noncommercial tests of ELISA should be restricted to laboratories with sufficient experience to set up the methods. Clotting assays are used for determination of the LA [24].

APA sy and aPLs in Reproductive Failure

Original classification criteria for APA sy, primary or secondary, also known as Hughes syndrome, was proposed at an international consensus workshop in Sapporo in 1998 [25] (table 1). APA sy laboratory criteria are the presence of serum anti-CL antibodies IgG and/or IgM isotypes on two or more occasions at least 6 weeks apart, and the presence of LA. Also the presence of antibodies to phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidyl-ethanolamine, phosphatidylcholine, phosphatidic acid, and the presence of antibodies to other coagulation-related proteins, e.g. prothrombin or thrombomodulin, are respected in reproductive failure, and can contribute to the definition of APA sy. Clinical criteria contain arterial or venous thrombosis, thrombocytopenia, and fertility failure (unexplained consecutive miscarriages with anatomic, genetic, or hormonal causes excluded), or one or more unexplained deaths of a morphologically normal fetus at or after the 10th week of gestation, or one or more premature births of a morphologically normal neonate at or before the 34th week of gestation associated with severe

preeclampsia or severe placental insufficiency, and three or more unexplained consecutive spontaneous abortions before the 10th week of gestation. One laboratory symptom (e.g. presence of aPLs) and one clinical symptom (e.g. repeated pregnancy loss) are sufficient for the definition of APA sy. An evident association with clinical symptoms of APA sy only positive levels of aCLs and/or LA are included in the formal criteria required to confirm definite APA sy.

Patients with APA sy have evidence of persistent coagulation activation: there is an increased plasma concentration of markers of thrombin generation (the prothrombin fragment F1-2 and fibrinopeptide A). What is less clear in thrombosis and miscarriage is the pathogenic role of the heterogeneous autoantibodies that characterize the fertility failure [26]. Koike [27] explains the relationship between aPLs and complement in vivo by complement activation, which is required for the creation of aPLs. According this hypothesis aPLs can activate complement in the placenta, generating factors which can mediate placental injury and lead to fetal loss and growth retardation. aPLs may also cause fetal loss in animals, possibly through interference with implantation first [28].

Immunological mechanisms of fertility failure as e.g. spontaneous abortion and fetal death have been the source of a number of recent reviews [e.g. 29–33]. The immunological causes of pregnancy loss may be classified as alloimmune, isoimmune, or autoimmune. The triad of deep vein thrombosis, recurrent abortion, and LA was first described by Soulier and Boffa [34].

Generally speaking, definition of the obstetric APA sy establishes associations between aPLs and thrombosis, fetal loss and thrombocytopenia, but very often only two symptoms are found, reproductive failure and aPLs. APA sy without another autoimmune disease is called primary APA sy. From an obstetric view, criteria for APA sy contain the presence of aPLs and repeated reproductive failure. Pregnancy loss is now categorized into at least three developmental periods [35]. The preembryonic period lasts from conception through to the beginning of the 5th menstrual week, the second up to the 14th week of pregnancy, and the last period up to the 34th week of pregnancy. Even earlier, in 1987, the group of El-Roeiy et al. [36] reported that women undergoing in vitro fertilization (IVF) demonstrated a surprisingly high incidence of aPLs abnormalities which appeared to reduce their chance of pregnancy with IVF. The presence of various kinds of elevated aPLs (table 2) can reduce the chance of spontaneous conception and/or conception with IVF [37–39]. Some laboratories test not only for anti-CL and antiphosphatidylserine antibodies, but for up to seven or eight antiphospholipids such as phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylcholine, phosphatidic acid, and anti-cofactor β2-GPI. For IgG, IgM and IgA isotypes [e.g. 38]. In the diagnosis of the APA sy, the persistence, isotype, and titer of the autoantibodies are an important consideration,

because transient low titers of e.g. anti-CL antibodies occur in up to 5% of healthy people without any clinical symptoms. Otherwise, mechanisms by which aPLs induce thrombosis and pregnancy loss include endothelial activation, interference with placental anticoagulant protein I (annexin V), and by anti-β2-GPI antibodies, induction of tissue factor or monocyte chemoattractant protein I, and inhibition of the anticoagulant function of activated protein C by autoantibodies.

Reproductive Failure and Neuroendocrine-Immunological Association

Reproductive failure in general demonstrates an unusual incidence of immunological abnormalities [40–43], as the result of neuroendocrine-immune associations, which are very often defective in infertile women (of course, andrological factors and clear genetic factors are excluded). Patients with three and more full IVF processes are highly selected women (repeated hormonal stimulations, repeated pick-ups and embryo transfers accompanying hormonal treatment). We also proved in our last study [43] in 120 women (78 infertile patients selected according to repeated IVF and repeated early pregnancy loss) significant autoantibody abnormalities in contrast with 42 healthy fertile women. We found a significant association between positive antiprothrombin and antibody elevation of three (against ph-inositol, ph-serine, ph-glycerol) and more autoantibodies against phospholipid-binding proteins in patients (54/78) with two and more repeated pregnancy losses after IVF. A high incidence of antizonal antibodies (69/78) in the same group of patients was observed, too. But, in countless studies in the literature, similar observations in reproductive failure have now been demonstrated as well. It is not only my hypothesis that the majority of women with repeated reproductive failure, that is repeated spontaneous miscarriages after spontaneous or artificial conception, or patients with preeclampsia, or HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count) or women with endometriosis, also called disease caused by immunological disorders [e.g. 44, 45], have an unusual incidence of immunological abnormalities. Patients with endometriosis stage I–II were associated with higher serum and peritoneal fluid levels of aPLs against ph-inositol, CL, ph-ethanolamine, and β2-GPI than those with stage III–IV. Cell and autoantibody abnormalities are considered as a defect of immunological coordination of maternofetal transplant relationships, natural cell suppressor activities fail. Latent ‘immune dysfunction’ starts before pregnancy and contributes to the immunological cause of infertility. In our view in the management of reproductive immunology in the obstetric laboratory it is quite sufficient to examine antibodies against seven different phospholipids (in IgG, IgA, IgM), sperm and

zona pellucida antibodies [e.g. 46]. We believe that autoantibody abnormalities contribute directly to spontaneous or IVF failure (on membranes of direct ovum-sperm fertilization, or in implantation membrane space of early embryo, in trophoblast cells or in placenta vessels, where thrombophilic properties of aPLs to endothelial or platelets may start direct microthrombotization in microcirculation). Women with reproductive failure and obviously patients at our IVF centers with repeated reproductive losses are patients of a highly selected group, with numerous neuroendocrine-immunological abnormalities. The autoantibody and/or lower hormonal and/or key cytokine levels as increasing of IL-1β, IL-6 and IL-10, IL-15 with a suggested role in fertilization, embryo implantation and pregnancy maintenance may be under neuroendocrine-immunological control at the mucosal site of the female and male genital tract [47].

aPLs, thrombosis and fetal loss can also be caused by genetic mutation involving factor V (factor V Leiden), prothrombin, or methylenetetrahydrofolate reductase, or by deficiencies of protein C, protein S, or antithrombin III. Concomitant presence of two or more prothrombotic risk factors may increase the risk. Approximately 15% of women with recurrent pregnancy loss have APA sy [32].

Management of aPLs, thrombosis and miscarriage of the obstetric APA sy aPLs is now recognized as representing the most frequent acquired risk factor for thrombophilia and as being a treatable cause of pregnancy loss. The widespread use of tests to detect aPLs, specialists such as rheumatologists, reproductive immunologists, hematologists and obstetricians make common treatment decisions regarding the consequences of positive test results and the clinical status of women who plan to become pregnant.

Clinical manifestations in APL sy make the collaboration of clinical and laboratory specialists in the sharp formulation of strategies for clinical management necessary. Therapeutic regimens are guided by the results of aPLs and all hematocoagluation examinations in connection with thrombosis.

Recurrent miscarriage in patients with APA sy, results of a controlled clinical trial indicate a better outcome in women treated with a low dose of unfractionated heparin twice daily plus aspirin than in those given aspirin alone [48]. Due to a very complicated process by which aPLs induce thrombosis and fertility failure, consideration for the treatment involves new anticoagulant and antiplatelet drugs, statins, antimalarial agents, interleukin-3, complement inhibitors, peptide competitors, anti-idiotype therapy, monoclonal antibodies, immunoabsorption procedures, and vaccination [32]. Today there is also some good experience with only heparin or fraxiparin, and aspirin combined with very low doses of corticosteroids [e.g. 49, 50]. Antioxidant diets are recommended, too. A very detailed algorithm for the pharmacological treatment of

Table 1. Clinical associations with aPLs

Primary antiphospholipid syndrome (APA sy I)
Venous or arterial thrombosis
Presence of ACA, or other aPLs, or β2-GPI
Infertility failure
Secondary antiphospholipid syndrome (APA sy II)
Rheumatic or connective tissue disorders + symptoms of APA sy I (autoimmune disorders)

Table 2. aPLs against the phospholipids in the literature [e.g. 37–39, 42, 43, 49]

CL, CL + cofactor β2-GPI also called apolipoprotein H
LA
Phosphatidylserine
Phosphatidylinositol
Phosphatidylethanolamine
Phosphatidylcholine
Phosphatidic acid
Phosphatidylglycerol
Annexin V in connection with phosphatidylserine

women with APA sy is shown in Derksen et al. [33]. For the management of obstetric APA sy, the wide availability and use of well-characterized and well-standardized aPLs assays and calibrators are essential. Periodic interlaboratory comparisons and quality control should remove doubts about clinically relevant cutoff values.

Autoantibody production is commonly associated with particular HLA class II phenotypes as we know of the phenotypization of patients, e.g. with SLE (HLA-DR7 and HLA-DR4). We did not confirm the association between aPLs and particular HLA alleles and HLA-linked epitopes in patients with primary APA sy. Formation of autoantibodies in recurrent miscarriage patients might be an epiphenomenon secondary to a T helper type 1 cytokine response against trophoblast, which might play a role in the pathogenesis of recurrent pregnancy loss [42]. We also determined six kinds of aPLs in ovulatory cervical mucus, the majority of which in very low levels, except for IgA-aPLs-inositol. We found very high interindividual differences in aPLs in seminal plasma. IgA-aPLs-ethanolamine and -phosphatidylglycerol, and IgG-aPLs-*L*-serine and -phosphatidylglycerol predominated in seminal plasma. Extremely high IgA levels of aPLs in six kinds of phospholipids, except CL, were found in the man of an

infertile couple (his wife had nine spontaneous miscarriages). The passage of abnormal aPLs levels from seminal plasma to the site of conception during often repeated, unprotected intercourse is supported by these findings, too [51].

We also compared levels of β2-GPI antibodies with six different aPLs in patients with primary APA sy, with preeclampsia, with autoimmune diseases, with diabetes mellitus and in a group with physiological pregnancy. The fact is that positive levels of aPLs against β2-GPI in IgA are more frequently associated with the diagnosis of APA sy, preeclampsia, and autoimmune disease than in pregnant patients with diabetes mellitus. Very high interindividual differences in aPLs (against ph-inositol, ph-*L*-serine, CL, and β2-GPI in IgG and IgA) were found in sera from women with pregnancy complicated by APA sy, preeclampsia and autoimmune disease. Pregnant patients with diabetes mellitus had higher serum levels of aPLs to ph-glycerol, ph-inositol, ph-serine, and β2-GPI. Positivity predominates in the isotype of IgG. Serum levels of anti-β2-GPI could serve as a better prognostic marker in complicated pregnancy than the standard panel of seven different aPLs. Detection of anti-β2-GPI is proposed as a first step of the screening for aPLs [52].

Conclusion

Long-lasting primary infertility or repeated pregnancy loss after spontaneous and/or artificial fertilization should be examined in antiphospholipid abnormalities as well. Laboratory information of aPLs potentially improves the chance of female conception through appropriately directed therapy. On the other hand, adequate treatment reduces risks during pregnancy when the patients conceive. Routine aPLs testing is recommend in all women who had two or more miscarriages. What is the best test to identify obstetric patients at risk? Which eight various aPLs including LA should be recommended? Which one is the best for reproductive failure? aPLs are involved in mediating clinical complications rather than simply being markers of a clinical syndrome. In my view all kinds of aPL examinations should be used, owing to the fact that the immunological system is highly flexible. Negative antibodies against e.g. CL and highly positive antibodies e.g. against ph-serine at the same time and in the same infertile women have to be taken into account. The patient is put at risk of spontaneous miscarriage. The detection of aPLs is very useful; pathological levels of aPLs may indicate not only obstetric problems, but e.g. an autoimmune disease which was latent up to the time of laboratory examination. Today, we have a panel of eight different phospholipids for the diagnosis of APA sy. But there are still open questions. Which one is the best biological marker for the APA sy? The diagnosis of APA sy requires the presence of both clinical and biological

features. Concerning pregnancy loss, there is general agreement that IgG antibodies against CL, against ph-serine, ph-ethanolamine, β2-GPI, ph-inositol at moderate or high titer and/or LA identify the patients with a greater risk of future obstetric complications than low-titer IgG or IgM of the same phospholipid factors.

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Oxidative Stress and Autoimmune Response in the Infertile Woman

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Abstract

There is convincing evidence that the establishment of a chronic inflammatory response, together with the presence of a local oxidative environment, could play an important role in the etiology and the progression of several human diseases. In the reproductive system, pathologies such as endometriosis, polycystic ovary syndrome, tubal obstruction, preeclampsia and recurrent abortions are related to the presence of inflammatory cytokines (TNF-α, IFN-γ, IL-1) and to high levels of free radicals that may damage biological molecules, such as lipids, proteins or DNA. Membrane lipids become oxidized and some of their products (malondialdehyde, acetaldehyde, hydroxynonenal) chemically modify proteins. These modified proteins consequently can change their function, antigenicity and therefore become implicated in immunological deleterious reactions associated with inflammatory and/or autoimmune injury. An altered protein function and the presence of circulating autoantibodies to new epitopes, such as malondialdehyde bound to proteins, could block some membrane surface antigens with a receptor function in the reproductive system. This explains how sperm capacitation, oocyte fertilization, or embryo implantation may be inhibited as a consequence of oxidative stress and chronic inflammatory conditions.

Introduction

The term oxidative stress is widely used and it refers to the situation of a serious imbalance between the production of reactive oxygen species (ROS) or reactive nitrogen species (RNS) and the antioxidant defense. ROS [e.g. superoxide anion, hydrogen peroxide (H_2O_2) and hydroxyl radical] and RNS (e.g. nitric oxide, NO) are unstable because of the unpaired electrons capable of initiating oxidation. These free radicals or molecules are highly reactive and they oxidize membrane phospholipids, proteins, carbohydrates or DNA.

Oxidative damage to proteins can occur by direct attack of ROS/RNS upon them, or by 'secondary damage' attack by end products of lipid peroxidation, such as malondialdehyde (MDA). Several ROS attack amino acid residues in proteins to form products with carbonyl groups. Protein oxidative modifications can affect antigen immunogenicity and the function of receptors, enzymes, transport proteins and signal transduction pathways.

Phagocytes are the first line of defense against invading pathogens or non-self structures, and they produce a battery of highly reactive oxidizing agents with microbicidal ability, which are also harmful for the nearby tissues, particularly under certain inflammatory circumstances. These cells produce superoxide by one-electron reduction of oxygen (O_2) at the expense of NADPH. Normally most tissues possess sufficient amounts of protective enzymatic [superoxide dismutase (SOD), catalase, glutathione peroxidase] and nonenzymatic (thiols, ascorbate, α-tocopherol) antioxidants that decompose most of the injurious oxidizing agents. However, the uncontrolled overproduction of ROS, together with an inefficient regulation of the immune system activation, results in a substantial oxidative damage to cells and tissues.

Radical scavenging systems prevent cell damage by ROS. The enzyme SOD catalyzes the conversion of superoxide radical to H_2O_2 and O_2. H_2O_2 is usually removed in aerobes by two types of enzyme: the catalase and glutathione peroxidase. The catalase directly catalyzes decomposition of H_2O_2 to ground-state O_2. Glutathione peroxidase removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced glutathione. One of the highly reactive microbicidal oxidant formed is hypochlorous acid (HOCl), which is produced by the myeloperoxidase-catalyzed oxidation of Cl^- by H_2O_2. Other reactive oxidants are hydroxyl radical (OH^-), produced by the reduction of H_2O_2 by Fe^{2+} or Cu^+ and peroxynitrite ($ONOO^-$) formed by the reaction between superoxide and NO.

A feature of free radical reactions is that when free radicals react with a molecule that contains no unpaired electrons, the product is another free radical. This gives rise to a very large variety of highly reactive free radical species. The sequence of events, in which radicals react to generate other radicals, is known as free radical chain reaction, and terminates only when a free radical reacts with another molecule with an unpaired electron (a free radical or a transition metal).

Redundant Loop Oxidation/Inflammation and Autoimmunity

The protective immune response involves the activation of T helper 1 (Th1) cells, macrophages and other phagocytic leukocytes, with the subsequent release of proinflammatory cytokines (TNF-α, IL-1, IL-6, IL-12), as well as ROS and NO. In a normal immune reaction to infections or antigens, once the pathogen or

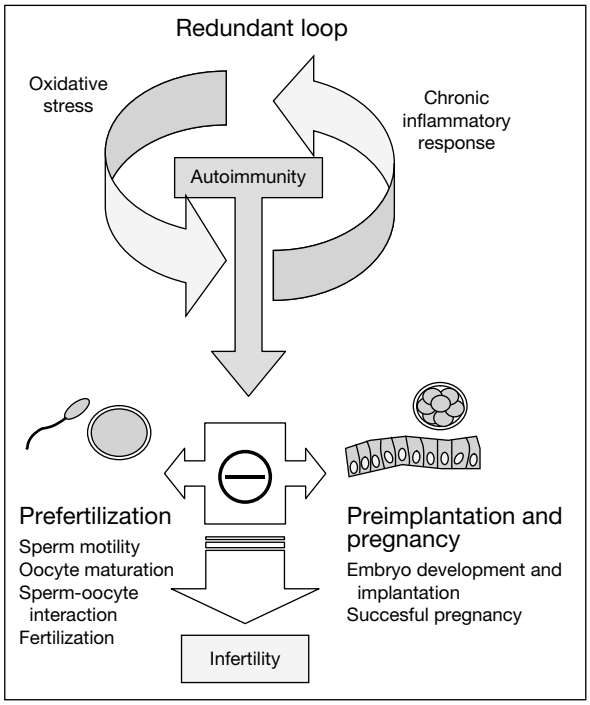

Fig. 1. Redundant loop between oxidative stress and chronic inflammation mediates oxidatively modified self-antigens and activation of autoimmune mechanisms. We propose that the synergic action of oxidative stress and inflammation may be the underlying cause of many pathological conditions in the female reproductive tract leading to infertility or pregnancy loss.

the antigen has been destroyed, there is usually a reversal of the inflammatory changes. However, if the pathogens or antigen are not completely eliminated, or the tissue injury continues, chronic inflammation can result. It is known that chronic inflammation is associated with enhanced production of reactive metabolites of O_2 and nitrogen. Both ROS and NO have been shown to modulate the inflammatory response. This enters in a redundant loop between oxidative stress and chronic inflammation (see fig. 1).

Intracellular oxidative stress is thought to activate, via several intermediate reactions, the proinflammatory nuclear transcription factor-kappa B (NF-κB) that is transported into the nucleus where it activates the transcription of a variety of genes known to be important in the inflammatory response [1]. It is well established that inflammatory cytokines, such as TNF-α, mediate apoptosis by the activation of multiple signals, partly mediated by the generation of ROS, which in turn are potent NF-κB activators.

Pathogen-induced inflammation destroys cells, either by the direct cytolytic activity of the infectious agent or by immune mechanism directed against the pathogen. This may lead to release of self-antigens and subsequent activation of autoimmune responses. The pathological consequences of autoimmunity usually become evident when autoimmune responses directed against self-antigens become chronic. There is now considerable evidence that neoself-epitopes generated in oxidatively modified lipids and proteins are important and even dominant antigens that drive the immune response. Our hypothesis is that oxidative stress synergically with chronic inflammation mediates an autoimmune process.

An imbalance in oxidation-reduction reaction status, with a consequent production of high amounts of free radicals, together with inflammatory events, may play an important role in the etiology of certain diseases: atherosclerotic disease [2] and multiple sclerosis [3]. Several lines of evidence suggest that the chronically inflamed tissues are subjected to substantial oxidative stress. Positive immunostaining for oxidative modifications in protein residues, such as nitrotyrosine, have been reported in encephalomyelitis brain samples [4]. In fact ROS and NO play a role in the pathophysiology of inflammatory diseases, such as the inflammatory bowel diseases [5], chronic obstructive pulmonary disease [6] and several diseases of the female reproductive tract, such as endometriosis.

Oxidative and Inflammatory Factors That Cause Female Infertility

Oxidative Stress

Endometriosis is a multifactorial disease that has been studied by several authors. There is evidence that immune factors and inflammatory events are involved in its pathophysiology. Oxidative stress is also proposed as a cause of the disease and the excess of free radicals plays a key role in human reproductive failure. Evidence of increased free radical formation is observed in the peritoneal cavity and ectopic tissue of endometriosis patients [7, 8]. Ectopic endometrium cells may be a possible source of oxidized lipid and proteins in the peritoneal cavity by diffusion or as a result of induction by tissue macrophages. Endometriotic implants could promote a prooxidant environment in the peritoneal cavity of women with endometriosis [8]. Several studies in endometriosis show evidence of increased cellular lipid peroxidation and accumulation of an end product of lipid peroxidation, MDA. On the other hand, women with peritoneal endometriosis also have lower levels of the antioxidant vitamins E and C [9].

Macrophages, through the scavenger receptors, play an important role in the uptake and removal of oxidatively modified proteins and phospholipids, which can be formed as a result of inflammatory processes. Peritoneal macrophages

from women with endometriosis produce an excess of NO. Wu et al. [10] found increased NO and eNOS activity in eutopic and ectopic endometrial tissue of endometriosis. In turn, activated macrophages contribute to the proinflammatory cytokine production enhancing the inflammatory reaction [8].

Chronic Inflammation

Chronic inflammatory processes are a common feature in several female reproductive pathologies: endometriosis [11], polycystic ovary syndrome (PCOS) [12], tubal obstruction [13], preeclampsia [14] and recurrent abortions [15]. Normal pregnancy requires a shift from a classical Th1 to a Th2 response [16, 17]. Not only repeated pregnancy loss, but other pregnancy complications as well, such as preeclampsia/eclampsia and unexplained recurrent spontaneous abortion, are associated with Th1 dominance [18–20].

Humans show complex Th1/Th2 profiles [21] and there is no consensus on whether a shift towards Th2 immunity is, in fact, a prerequisite for normal pregnancy [17, 22]. In pregnant mice, exposure to stress leads to elevated levels of TNF-α and IFN-γ cytokines. Upregulation of these two cytokines is observed in female mice injected with IL-12 during early gestation [23]. An increase in peritoneal inflammation, as evidenced by elevated peritoneal fluid cytokine levels, is well established in women with endometriosis [24]. Several investigators have shown that TNF-α concentrations are elevated in the peritoneal fluid of endometriosis, and that high concentrations correlate with the stage of the disease [24]. TNF-α induces other proinflammatory cytokines, such as IL-1, IL-6, and additional TNF-α [24, 25]. Although it is uncertain whether the elevated cytokine levels or inflammation are the cause or the result of the disease, it is clear that these cytokines may have profound effects on reproduction. In endometriosis some evidence shows that the lymphocytes may contribute to the cytokine production of peritoneal fluid, and are involved in cellular growth in ectopic implants [26] and inflammatory reactions [27].

In preeclampsia, intravascular excessive inflammatory response is the cause of the clinical syndrome and involves lipid peroxidation and activation of leukocytes in the intervillous space, resulting in endothelial injury [28]. In this pathology, high levels of IL-12 [29] released by monocytes/macrophages in response to several pathogens induce the early production of IFN-γ by Th1 cells and natural killer (NK) cells. IL-12, in synergy with other proinflammatory cytokines, IL-1, TNF-α and IFN-γ, markedly enhances the chronic inflammation in this disease [30].

Autoimmunity

Genetic, hormonal and environmental factors and immunity dysfunctions have a role in the pathogenesis of autoimmune diseases [31]. In the reproductive

system, a common disorder, pelvic endometriosis, a benign chronic inflammatory disease, is considered to be an autoimmune disease, because it fulfills most of the classification criteria for such disorders [31, 32].

Autoantibodies have been described in most of the female reproductive diseases. Approximately 20 years ago, Weed and Arquembourg [33] hypothesized that an autoimmune mechanism might explain poor reproductive outcomes in some women. A decade later, Mathur et al. [34] were the first to describe in patients with endometriosis autoantibodies which recognize endometrial antigens that are candidates for the autoantigens responsible for the immune response [35, 36]. Gleicher et al. [37] noted that a significant proportion of women with endometriosis (40–60%) have elevated autoantibody titers when tested against a panel of autoantigens. We have reported the presence of antiendometrial antibodies in women with tubal factor, ovarian failure and endometriosis [38] and, interestingly, antiendometrial antibodies were correlated to the severity of endometriosis [39]. Some authors have detected autoantibodies to antigens relevant in reproduction [40], such as zona pellucida antibodies, which have been described in patients with unexplained infertility. These antibodies are suspected of interfering with sperm binding [41].

Subclinical and persistent infections in the fallopian tubes are a significant danger to future fertility. In *Chlamydia trachomatis* infection, the parasite produces high levels of heat shock protein 60 (c-HSP60), which contributes to the development of tubal occlusion and adverse pregnancy outcome. Human HSP60 (h-HSP60) shares a 50% amino acid sequence homology with c-HSP60; therefore, chlamydial infection can induce autoantibodies to h-HSP60 [42]. There has also been a report of anti-h-HSP60 in women with endometriosis, in whom the peritoneal fluid mononuclear cells produce great amounts of inflammatory TNF-γ in response to HSP60 stimulation [43].

Abnormalities in the process of apoptosis have been postulated to play a role in autoimmune diseases [44]. Several studies have demonstrated that endometrial cells from eutopic and ectopic endometrium from women with endometriosis have altered apoptotic mechanisms [24, 45, 46, 47]. Thus, endometrial self-antigens persist in the peritoneal cavity.

The reduced NK activity during endometriosis could at least in part explain the increased autoimmune reactivity associated with the disease: in the peritoneal cavity, NK cells might be less effective in killing autologous dendritic cells loaded with endometrial self-antigens, facilitating their presentation to autoreactive T cells and the production of autoantibodies [31, 48, 49].

The presence of specific antibodies to phospholipid antibodies [50], thyroglobulin antibodies [51], antiprothrombin antibodies [52], and antilaminin-1 antibodies [53] have been associated with spontaneous pregnancy loss and with other infertility pathologies [54]. Antiphospholipid antibodies

that have detrimental effects on pregnancy are directed to phospholipids and phospholipid-binding proteins. Women with preeclampsia have elevated titers of antiphospholipid antibodies and their placenta is under oxidative stress. This is evidenced by abnormally elevated tissue levels of lipid peroxide breakdown products, increased expression of $ONOO^-$ [55], increased levels of oxidized proteins [56], and increased trophoblast generation of superoxide [57]. Autoantibodies to oxidatively modified proteins have been detected in preeclampsia [58]. Antigenicity is attributed to specific modified epitopes [59]. It has been suggested that such an antibody response may be a reflection of the underlying state of lipid peroxidation [60].

Recent studies in endometriosis have pointed out that Ox-LDL is potently antigenic, proinflammatory, and alters the function of a number of cell types [8]. In PCOS, the production of antiovarian antibody may be related to an inflammatory reaction with abnormal production of cytokines and overexpression of class II antigens [3, 61]. Our results show that antiendometrial and anti-MDA-modified protein antibodies are significantly higher in sera from PCOS patients than in sera from fertile women (data not published).

Oxidative and Inflammatory Factors in Reproductive Function

Several authors have reported the effects of ROS/antioxidants [62] and inflammatory cytokines [63] on the female reproductive function. Excessive concentrations of free radicals and inflammatory cytokines in biological fluids (mainly peritoneal fluid or follicular fluid) exert cytotoxic effects on germ cells, embryo and its early development [64].

Gametes

Low levels of lipid peroxidation are important for normal sperm function, such as sperm activation, capacitation, acrosome reaction and sperm binding to the zona pellucida [65, 66]. However, increased levels of ROS in the peritoneal fluid from women with idiopathic infertility may be detrimental to sperm function. As a consequence, DNA fragmentation [67], lipid peroxidation [68] and oxidative protein damage [69] occur. Lipid peroxidation in spermatozoa results in decreased membrane fluidity and a reduced fertilization ability [70], or leads to a nonphysiological premature capacitation and acrosome reaction [68]. Also, the ability of capacitated sperm to fuse to oocytes depends on the presence of nonoxidatively damaged sperm plasma membrane proteins [71, 72]. The source of ROS may also be produced by immature spermatozoa or by other invading cells in seminal plasma (neutrophils, lymphocytes) in leukocytospermia. In

semen samples from infertile men, we detected high levels of oxidized proteins and a reduced sperm motility (pers. commun.).

Asymptomatic chronic infection (bacterial or nonbacterial) of the reproductive tract is associated with an increased ROS generation, an increase in proinflammatory cytokine expression (TNF-α, IFN-γ, IL-6, IL-8) and the activation of humoral and cellular immune mechanisms that have been implicated in defective sperm motion and function [73].

Lipid and protein oxidative modifications may be recognized as neoantigens. Antisperm antibodies, in infertile couples, may influence fertility by binding to the sperm surface and this can affect sperm motility, acrosome reaction, penetration into cervical mucus, binding to the zona pellucida and sperm-egg fusion [74].

It has been reported that cumulus cells are involved in the cytoplasmic maturation of oocytes and protect oocytes from cell damage caused by oxidative stress [75]. The generation of ROS, although an important physiological process, may also induce pathological events in the ovary.

Preimplantation Embryo and Endometrium

Oxygen free radicals may also be involved in embryonic development in vivo [76]. Under extreme oxidative conditions or if the antioxidant protective mechanisms of cells are compromised, embryo injury or death may occur [77]. Early mammalian embryos are susceptible to damage from ROS, as they increase in the medium when cultured in vitro [77]. The exposure of pregnant female mice to LPS (through intraperitoneal injections) leads to preimplantation embryonic loss and also causes severe damage to the placenta or the endometrium [78]. In our studies, we determined that LPS produces a decrease in the number of preimplantation embryos in a concentration-dependent manner and this effect can be partially reverted or prevented by vitamin E [79].

TNF-α can have deleterious actions on oocyte maturation which compromise the development of the resultant embryo. While exposure of fertilized embryos to TNF-α does not inhibit development to the blastocyst stage, TNF-α increases the percentage of blastomeres undergoing apoptosis when exposure occurs in ≥9-cell embryos [80]. Genital tract infections or low doses of LPS may trigger the production of TNF-α, and these conditions are responsible for the production of abnormally developed preimplantation embryos in rats [78].

The human blastocyst forms and penetrates the endometrium which has become receptive to implantation. During the peri-implantation period, many molecules such as cytokines (IL-12, IL-18) [22], growth factors, hormones, and many cells such as NK [81], play key roles in the interactive communication between the endometrium and the embryo. Oxidative stress may impair fetal development indirectly by interfering with the maternal capacity to support

embryo-fetal development. The pregnancy loss rate in spontaneous pregnancies is about 15–19% of all pregnancies diagnosed [82].

Pregnancy/Abortion

Significantly low ROS levels and normal levels of Cu/Zn-SOD contribute to the maintenance of normal pregnancy. In contrast, high levels of ROS [83] and NO [84] are detrimental to fertility. The decrease in Cu/Zn-SOD expression and the increase in lipid peroxides in the decidua could be involved in spontaneous abortion [85].

Th2-type cytokines (IL-10, IL-4) have been proposed to be the normal profile in successful pregnancy, and a deficiency in these cytokines may lead to poor placentation, subnormal growth and possibly even fetal death [83, 86, 87]. In contrast, Th1 inflammatory cytokines (IFN-γ, TNF-α, IL-2) are indeed deleterious to pregnancy [19, 63] and may lead to adverse effects on the conceptus either by direct embryotoxic activity or by damaging the placental trophoblast. Th1-type cytokines may also act by inducing NK and lymphokine-activated killer cells. NK activity has been unquestionably linked with spontaneous fetal resorption in mice [87].

Recent studies on cytokine defects and implantation failure include other cytokines playing important roles in abortion. IL-18 is seen as Th1-like in the presence of IL-12, which is present in the decidua, but in the absence of IL-12, IL-18 has Th2-inducing properties. IL-12 plus IL-18 would result in abnormal NK cell function and would induce abortion in preeclampsia [22]. T cell activation, persistent production of TNF-α and oxidative stress has been linked with miscarriage.

Concluding Remarks

The immune system is the natural defense against infectious agents and malignant tumors. Phagocytic cells, which are components of the innate immune response, are capable of generating large amounts of ROS. However, when antigens are not rapidly eliminated, persistent free radicals and inflammatory cytokines are continuously released causing tissue damage without any successful chance of tissue repair.

Some theories have been proposed for the pathogenesis of endometriosis, preeclampsia, recurrent abortion and other diseases related to fertility impairment. Several pieces of evidence of a direct relationship between oxidative stress and chronic inflammation in a redundant loop lead to oxidatively modified lipids and proteins in cells relevant to the reproductive system. This supports our idea that oxidation and chronic inflammatory events, in a free radical chain reaction,

cause cell damage and continuously release modified autoantigens. This activates the innate and adaptive immune system inducing autoimmune responses and antibodies to modified self-epitopes.

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Trophoblast Invasion and Placentation: Molecular Mechanisms and Regulation

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Abstract

Trophoblast invasion is a key process during human placentation. This event constitutes the basis of the conversion of the uterine spiral arteries, a process which allows an adequate vascular connection between the intervillous space and the maternal blood flow. Trophoblast invasion is transient, with stringent spatial and temporal control. Preeclampsia, a leading cause of maternal and fetal mortality and morbidity, is associated with decreased, shallow trophoblastic invasion. In this article, we review the molecular mechanisms of trophoblast invasion, and its mechanisms of regulation. Insights into the etiopathogenesis of preeclampsia will also be detailed.

Introduction

Implantation of the human egg occurs 6–7 days after conception. This process probably includes three stages. Apposition, the initial adhesion of the blastocyst to the uterine wall, usually occurs at the upper posterior wall of the uterus. Then stable adhesion of the blastocyst to the endometrium occurs. This event is followed by invasion of the decidua. At this moment, the embryonic pole of the blastocyst is oriented towards the uterine epithelium [1].

Trophoblast cells are derived from the trophoectoderm of the blastocyst. These cells adhere to the endometrium and initiate the process of implantation. During the first trimester of gestation, cytotrophoblast (CTB) stem cells reside in two types of chorionic villi, namely floating and anchoring villi. The CTBs can

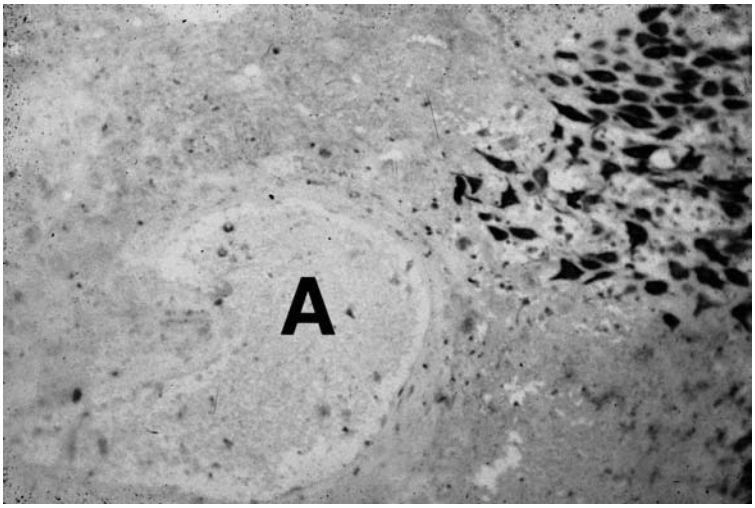

Fig. 1. Placental bed of a normal human pregnancy, 13 weeks of gestation. Intermediate trophoblasts (immunostained for cytokeratin, brown color, upper right part of the picture) invade the decidua close to a maternal artery (A).

differentiate according to two distinct pathways, giving rise to two trophoblast populations that are morphologically and functionally distinct [2–5].

Floating villi do not contact the uterine wall. In these villi, CTBs consist of a polarized epithelial monolayer, anchored to a basement membrane and surrounding a stromal core containing fetal blood vessels. These CTBs highly proliferate during the first trimester of gestation and differentiate exclusively by forming a multinucleated syncytium called syncytiotrophoblast (STB) that covers the villi. Floating villi are bathed by maternal blood and perform gas and nutrient exchange functions for the developing embryo. STBs also produce placental hormones and growth factors.

Anchoring villi contain CTB stem cells that enter both pathways of differentiation. In much of the anchoring villus, CTB fuse to form STB. At day 10, the blastocyst is completely embedded into the stroma of the uterus, and CTBs break through the syncytial layer and form multilayered columns of nonpolarized cells. Anchoring villi, via these columns, physically connect the embryo to the uterine wall and are the source of the most highly invasive CTBs, the intermediate trophoblasts [6–8], found in the pregnant endometrium (decidua) and the first third of the myometrium, collectively designated the placental bed (fig. 1). A subpopulation of the intermediate trophoblasts invades the uterine blood vessels (endovascular invasion; fig. 2), a process which establishes adequate perfusion

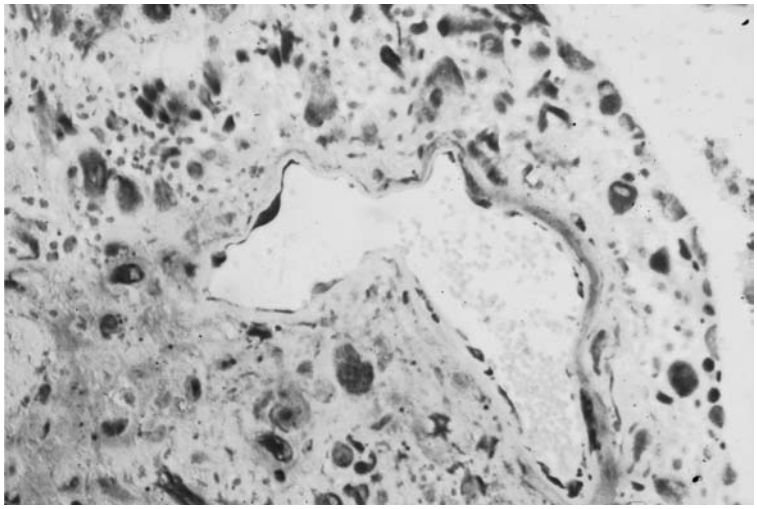

Fig. 2. Normal uteroplacental artery, 14 weeks of gestation. The arterial wall is invaded by trophoblastic cells (immunostained for cytokeratin) who replace the media and endothelium.

of the placenta [9,10]. The endovascular trophoblasts migrate to the uterine spiral arteries, where they replace the endothelial cell lining and the muscular and elastic layer of these arteries. This process, named conversion, occurs during the first trimester of pregnancy, and leads to low-resistance, high-capacitance blood vessels that allow for adequate blood flow during the pregnancy.

The process of anchoring villus formation and related CTB invasion of the uterine wall is extremely active during the first trimester of gestation. The morphology of the placenta is quite similar at the first and second trimester. During the third trimester, cell columns are no longer visible.

Many aspects of trophoblast invasion are thought to be similar to events that occur during tumor cell invasion, when an in situ lesion becomes an invasive carcinoma, i.e. when cells penetrate an epithelial basement membrane and invade the underlying stroma [11]. Like malignant tumor invasion of the host tissue, trophoblast invasion of the maternal uterus is a multistep process. It involves attachment of the trophoblastic cells to the extracellular matrix (ECM) components, degradation of the ECM and migration through the connective tissue defect [5]. However, unlike tumor invasion, trophoblast invasion of the decidua is precisely regulated, confined spatially to the uterus and temporally to early pregnancy.

Dysregulation of trophoblast invasion is associated with various pathological problems. Indeed, excessive invasion can lead to placenta accreta, increta or percreta [12] that constitute leading causes of postpartal hemorrhage.

Uncontrolled trophoblast invasion can also lead to choriocarcinoma with possible metastases [13].

On the other hand, inadequate trophoblast invasion is associated with preeclampsia. This entity is characterized by maternal edema, pregnancy-induced hypertension and proteinuria, and, in severe cases, eclampsia and abruptio placenta. Preeclampsia is associated with significant maternal and fetal death [12]. It is caused by limited trophoblastic invasion and a failure to convert the spiral arteries. This leads to the conservation of the reactivity of the maternal arteries to vasoconstrictive agents, resulting in placental hypoxia. Another entity associated with placental abnormalities and reduction of trophoblast invasion is intrauterine growth retardation.

In-depth understanding of the molecular mechanisms that underlie trophoblast invasion could help understand the etiopathogenesis of diseases like preeclampsia and intrauterine growth retardation. It could also constitute the basis for new innovative therapeutic strategies for these diseases. In this article, we review the molecular mechanisms of trophoblastic invasion.

Adhesion Molecules

Adhesion of various types of cells are mediated through specific cell surface receptors designated integrins. These receptors are heterodimeric αβ-type transmembrane glycoproteins. Their specificity of binding depends on the type of α/β combination. CTBs express integrins to adhere to ECM components, and modulate their integrin repertoire when they invade the mother tissues (table 1) [14, 15]. Examination of first trimester placenta [14, 16] and of an in vitro model of Matrigel invasion by CTBs [15] reveals that the invasion of CTBs leads to a decreased expression of adhesion receptors characteristic of CTB stem cells, and an increase in the expression of adhesion receptors that are characteristic of vascular cells.

Indeed, villous CTBs predominantly express the α6β4 integrin, a laminin receptor. In contrast, the invasive extravillous CTBs present with a differentially modulated integrin repertoire. Columnar CTBs express α6β4 in a nonpolarized fashion; more distal, placental bed-invasive CTBs express α5β1 integrin, a fibronectin receptor. Interstitial and endovascular CTBs also express α1β1, a laminin and collagen receptor [14, 15]. This profile of integrin expression is also observed on CTBs that invade Matrigel [15]. The importance of these adhesion molecules has been highlighted by in vitro experiments. Antibody perturbation of the interactions involving α1β1, type IV collagen and laminin inhibits CTB invitro invasion, whereas perturbing interactions between fibronectin and α5β1 promoted invasion [15].

Table 1. Expression of adhesion molecules and integrins in first trimester trophoblast [data from 14, 17, 23–26, 117]

	Zone I villous CTB	Zone II column CTB proximal	Zone III column CTB distal	Zone IV placental bed CTB interstitial	Zone V placental bed CTB endovascular	maternal ECs
α1	–	–	–	+	+	±
α4	–	+	+	+	+	±
β1	±	±	+	+	+	+
β3	–	–	+	+	+	+
β4	+	+	±	–	–	–
β5	+	–	–	–	–	–
β6	±	±	–	–	–	–
E-cadherin	+	+	±	±	–	–
VE-cadherin	–	+	+	+	+	+
αVβ3	–	–	–	+	+	+
VCAM-1	–	±	+	+	+	±
PECAM-1	–	–	+	+	+	+
NCAM-1	–	–	–	–	+	
CEACAM-1	–	+	±	±	±	
MCAM	–	+	+	+	+	
E-selectin	+	+	+	+	+	±
L-selectin	+	+	+	+	+	

Zone I = Monolayer of CTB on the trophoblast basement membrane; zones II and II = cell aggregate (column) formed by differentiating CTB; zone IV = interstitial trophoblasts that have invaded the maternal decidua; zone V = endovascular trophoblasts and placental bed CTBs.

The CTBs also undergo an epithelial to endothelial cell transformation. Indeed, they decrease E-cadherin, and increase endothelial-specific adhesion molecules, such as VE-cadherin, PECAM-1 and VCAM-1 [17–20]. They also express various vasodilatory and anticoagulative factors [21]. Functional studies using blocking antibodies reveal that αVβ3 and VE-cadherin enhance, and E-cadherin decreases CTB invasion [17].

Examination of placenta from the second and third trimester of gestation revealed similar patterns of integrin expression with only slight differences [16]. Indeed, during the second trimester, the α3 integrin subunit is detected on villous CTBs, columns and placental bed CTBs. Third trimester floating villi are characterized by persisting expression of the α6 integrin subunit, whereas β4 is not detected.

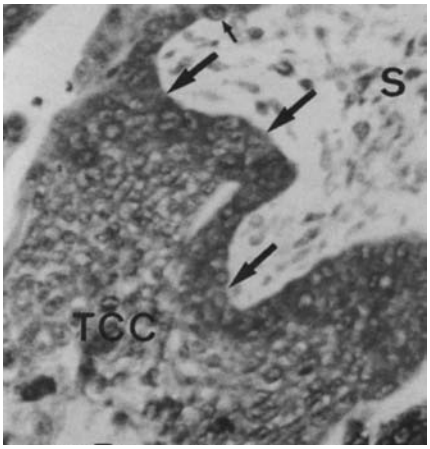

Fig. 3. Immunohistochemical localization of galectin-3 in a first trimester galectin-3 expression when polarized CTBs (arrows) differentiate to form the trophoblastic cell column (TCC). S = Villous stroma.

The focal adhesion kinase (FAK), which is implicated in signal transduction after binding of the ligand on integrins, is expressed by CTBs in all stages of differentiation [22]. Autophosphorylation on tyrosine 397 (Y397FAK) is only detected in a subset of invasive CTBs near the surface of the uterine wall. It appears to mediate CTB invasion, as antisense FAK induces a striking reduction of in vitro invasion [22].

Other adhesion molecules are differentially expressed during trophoblast differentiation and invasion. For instance, the adhesion molecule CEACAM-1 (CD66a) is not expressed in villous CTB and STB; it is strongly expressed in proximal columns, and is also observed in interstitial and endovascular trophoblasts [23, 24]. Human trophoblasts express functional L-selectin, a molecule that could play an important role during interactions between the trophoblasts and the uterine luminal epithelium [25]. Melanoma cell adhesion molecule (MCAM) is expressed by extravillous CTBs but not villous CTBs [26]. The expression of galectin-3, a mammalian lactose-binding lectin implicated during cancer progression [27], is downregulated during the first trimester of gestation, with minimal expression at 12 weeks when invasion is maximal [28]. Immunohistochemistry reveals that expression of galectin-3 is decreased when trophoblasts differentiate into cell columns [29], i.e. when CTBs acquire a migratory phenotype (fig. 3).

Placentas from preeclamptic patients are characterized by defective trophoblastic invasion and by abnormal switching of integrins. Indeed, placental bed CTBs present with a persisting expression of $\alpha 6\beta 4$ and absence of $\alpha 1$ upregulation, suggesting that $\alpha 6\beta 1$ integrin is expressed by placental bed CTBs as early as the late second trimester, a situation that does not exist in normal pregnancy until term [16]. Levels of plasma membrane Y397FAK are also

decreased [22]. Moreover, preeclamptic CTBs fail to mimic a vascular adhesion phenotype and retain expression of αVβ6 and E-cadherin, and fail to upregulate αVβ3, VE-cadherin, VCAM-1 and PECAM-1 [30]. In in vitro experiments, preeclamptic CTBs are also characterized by reduced invasion and α1 integrin expression [31]. This suggests that preeclamptic trophoblasts present with an altered expression of adhesion molecules, resulting in a reduced ability to invade the decidua.

Proteases

A large body of evidence demonstrates that trophoblast invasion is not due to passive mechanisms but is an active process. Indeed, trophoblasts from the first trimester of gestation are able to degrade the matrix by secreting proteases [5, 32–34]. Several types of proteases have been involved in this process, including serine proteases, cathepsins and matrix metalloproteinases. Secretion of these proteases begins already at the blastocyst stage [35, 36].

Human CTBs are able to invade amniotic membranes [37] and Matrigel invitro [38–40], and to digest ECM [5]. This behavior is clearly related to secreted metalloproteinases, as TIMP expression inhibits their invasiveness [37]. Gelatin zymography demonstrates that several metalloproteinases are uniquely expressed by first-trimester invasive CTBs, and not by second- and third-trimester trophoblasts [5]. Matrigel invasion by CTBs is clearly related to expression and activity of MMP-2 and MMP-9 (gelatinase B) [5, 41]. Expression and activation of MMP-9 peak during the first trimester of pregnancy at the moment when invasion is maximal [40–42].

Human extravillous CTBs acquire an invasive phenotype on Matrigel associated with a specific pattern of protease gene expression, including increased MMP-12, MT2-MMP, TIMP-2 and TIMP-3 expression, and decreased MMP-2 and TIMP-1 expression [43]. Increased expression of TIMP-3 by trophoblasts, simultaneous to that of MMP-9, provides a mechanism for controlling MMP-mediated invasion [44]. Trophoblast invasion seems also regulated by the production of MMPs and TIMPs by the maternal decidua [45]. Invasive trophoblasts produce stromelysin-3 during both, the first and third trimesters of pregnancy, but to a lesser extent during the latter [46]. STBs from the floating villi also express stromelysin-3 [46]. Plasminogen activator inhibitors or a function-perturbing antibody raised against uPA only partially inhibit CTB invasion in vitro [41]. Human trophoblast also expresses urokinase-type plasminogen activator receptor [47] that can bind active urokinase-type PA and concentrate proteolysis at the invading edge of the cells. Activity of this system is altered in preeclampsia [48]. Proteolysis of the thrombin receptor, protease-activated receptor-1 (PAR-1), is the predominant

thrombin receptor on invasive extravillous trophoblast cells and may play an important role during trophoblast invasion [49]. Cathepsin B and L may also be important during trophoblast invasion [50].

Examination of preeclamptic patients reveals that their trophoblasts are characterized by reduced in vitro invasion and MMP-9 expression [31] and activation [48], and secrete less cell surface PAI [48].

Regulation of Trophoblastic Invasion

Trophoblastic invasion is controlled by several factors. The trophoblasts should first differentiate to anchoring trophoblasts. This is partly attributed to the contact of the trophoblast with specific, still to be characterized decidual factors, via either paracrine stimulation or direct contact with the decidual ECM [51]. This view is somewhat complicated by a recent study that examines viable tubal pregnancies [52], showing that extravillous trophoblast differentiation [defined by expression of different molecules including integrins, E-cadherin, EGF receptor (EGF-R), Ki-67 and HLA-G] was similar, except for the fact that the columns were markedly longer in tubal pregnancies [52].

Steroids

Treatment of first trimester CTBs with progesterone decreases MMP-9 expression [53].

Extracellular Matrix

Type I collagen has the ability to stimulate gelatinase secretion by CTBs [54].

Hormones and Cytokines

Placental expression of chemokines is specific to either the stromal or CTB compartment of the villi [55], suggesting that they could participate in trophoblast differentiation and invasion.

Transforming growth factor-β produced by the decidua [56, 57], STBs and extravillous CTBs [58] induces trophoblast differentiation toward an anchoring phenotype including production of oncofetal fibronectin [59, 60].

The EGF-R is mainly expressed by villous CTB and STB [61], and surrounding decidual cells [62]. This receptor can bind EGF (expressed in uterine epithelial and decidual cells, and villous CTBs and STBs [63]), TGF-α (expressed in decidual cells and all trophoblast lineages [64]) and amphiregulin (expressed by STBs before 18 weeks of gestation [65]), and stimulate extravillous trophoblast proliferation [64, 66].

Production of LIF by postovulatory endometrium [67] could decrease trophoblast synthesis of human chorionic gonadotrophin (hCG), and increases oncofetal fibronectin, which suggests that it stimulates CTB differentiation towards an anchoring extravillous phenotype [68].

It has been shown that the hypoxia-inducible factor-1α (HIF-1α) increases the expression of several genes important for invasion. This includes TGF-β3, the expression profile of which is similar to that of HIF-1α [69, 70]. This molecule is an inhibitor of the invasive extravillous phenotype of trophoblasts [70]. Preeclampsia is characterized by persistence of TGF-β3 expression after the first trimester and trophoblasts are arrested at an intermediate immature phenotype [69, 70], suggesting that this anomaly is linked to the defective trophoblast invasion characteristic of the disease. Interestingly, in vitro antisense inhibition of TGF-β3 expression or inhibition of TGF-β3 activity with blocking antibodies induces the formation of columns of CTBs and restores their in vitro invasive phenotype [69].

Numerous hormones and cytokines are also able to modulate trophoblast invasion by modulating the expression or activity of adhesion molecules or proteases.

The cytokines IL-1 and IL-6 increase the expression of the α1 and α2 integrin subunits in CTBs [71]. Expression of integrin α1 and in vitro invasion of CTBs are reduced when interfering with the binding of VEGF ligands to the CTBs [72]. Leptin, IL-1α, IL-6 and TGF-β increase the expression of the α5 and α6 integrin subunits [73]. Insulin growth factor binding protein-1 (IGFBP-1), the major secretory product of the decidualized endometrium, binds the α5β1 integrin and stimulates cell migration [74]. Both IGF-II, which is produced by extravillous CTBs and IGFBP-1, synergistically enhance invasion of first trimester trophoblast cells [75].

MCAM expression of MCAM in JEG3 cells and trophoblast explants is increased in the presence of human decidual tissue through PKA and cAMP pathways [26].

In vitro invasion and collagenolytic activity of the cytotrophoblastic JEG-3 cells is stimulated by hCG through a cAMP-dependent pathway [76]. Human first trimester CTB in vitro invasion is upregulated by EGF [77] and hepatocyte growth factor (HGF) from the stroma of the villi [78]. Preeclampsia is characterized by a decreased expression of HGF by the stroma of the villi [78]. Protein kinase C activators such as phorbol esters increase in vitro trophoblast invasion [38] and secretion of MMP-9 [79]. The activity of MMP-9 is upregulated by leptin and IL-1α [73, 80], and it is known that IL-1 concentrations in embryo culture medium are correlated with success of in vitro fertilization procedures [81].

CSF-1 present at the fetomaternal interface [82, 83] binds to the M-CSF receptor (M-CSF-R, the product of c-fms) present on trophoblast cells since

the blastocyst stage, and, later, on extravillous CTBs [83–85]. It increases cell proliferation but not invasiveness, and increases expression of both MMP-2 and TIMP-1 [86]. The expression of M-CSF-R has been correlated with trophoblast invasiveness [62].

Immune System

The placental bed contains a large population of immune cells, including uterine natural killer (NK) cells [87]. The survival of the conceptus depends on its acceptance, or nonrejection, by the maternal immune system [88]. Thus, the maternal immune system likely interacts with the process of trophoblast invasion.

For instance, HLA-G, a major histocompatibility tissue-specific antigen of low polymorphism, is expressed by invasive extravillous CTBs, including interstitial trophoblasts, placental bed giant cells and endovascular trophoblasts [89]. HLA-G1 reduces both CD8+ and CD4+ T cell reactivity and decreases innate immunity of uterine NK cells [90, 91]. This could contribute to protecting invasive CTBs from attack by the uterine NK cells [92] and enables these cells to invade the uterus. In preeclampsia, expression of HLA-G is reduced or absent in extravillous CTBs [31, 93–95]. This could lead to a defective recognition of infiltrating CTBs by uterine NK cells, resulting in defective invasion and spiral artery conversion [96, 97].

A recent study demonstrates a specific profile of chemokine expression in the stromal compartment and trophoblast population of the chorionic villi, which could participate, for instance, in the recruitment of the resident macrophage (Hofbauer cell) population of the villi, CTB differentiation and invasion [55].

Transcription Factors

It seems that the invasive phenotype of CTBs results from the coordinated expression of gene products implicated during trophoblast invasion, such as integrins and MMPs, thus suggesting superior control by nuclear transcription factors. On the other hand, external stimuli induce intracellular signal transduction that could result in the modulation of expression and/or activity of transcription factors.

Using a transgenic mouse model system carrying homozygous gene mutations, several master regulators of trophoblast differentiation have been described [98, 99]. These transcription factors include Hand1, Mash2, Id2, E-factor, I-mfa, GCM1 and Sta13. Descriptive studies have shown that several of these factors are also expressed in the human placenta, suggesting that the mechanisms controlling trophoblast differentiation could be similar in mice and men. A chapter in this book is dedicated to this topic.

Several transcription factors have already been examined. For instance, the promoter site of MMP-1, MMP-3 and MMP-9 is able to bind the AP-1

complex, a heterodimer of Jun and Fos, which is thought to be important during the mediation of signals that lead to increased MMP expression. Indeed, the AP-1 complex is implicated in the MMP-1 response to IL-1, TNF and TGF-β [34, 62] and in the MMP-3 regulation by PDGF and TGF-β [34, 62]. Both Fos and Jun are highly expressed in human trophoblast [100]. Moreover, the effects of EGF on trophoblast proliferation and differentiation depend on modulation of c-fos and c-jun expression [101]. Jun-B is essential for the establishment of vascular connections with the maternal circulation during mouse placentation [102].

The transcription factor c-myc activates telomerase by interaction with the hTERT (telomerase reverse transcriptase) promoter [103] and seems essential for progression of the cell cycle. The expression of c-myc correlates with early trophoblast proliferation [104]. Moreover, expression of c-myc colocalizes with expression of c-sis, which encodes for the β chain of PDGF, and is thought to participate in the control of proliferation of trophoblasts [105].

An important transcription factor for placentation is the HIF-1 protein complex [106, 107], which binds a short DNA motif located in the 5′-flanking regions of various hypoxia-induced genes [108]. HIF-1 binds DNA as a heterodimer composed of 2 subunits, the constitutively expressed HIF-1β (aryl hydrocarbon receptor nuclear translocator, ARNT) and HIF-1α, which is induced in hypoxic conditions and is quickly degraded by the proteasome under normoxic conditions through an interaction with the von Hippel-Lindau tumor suppressor protein (pVHL) [109]. Trophoblasts grown under hypoxic conditions are used to model the cellular environment of normal and preeclamptic gestation [110, 111]. Indeed, normoxic CTBs differentiate towards an invasive phenotype, which allows them to invade Matrigel [5, 41], as observed in invasive intermediate trophoblasts that are in a hypoxic environment. Hypoxic conditions (2% O_2) induce trophoblast proliferation, increase fibronectin synthesis, expression of α5 integrin, gelatinase A activity, and inhibit invasion, due at least in part to their inability to switch their integrin repertoire, such as induction of α1β1 [70, 110]. This is thought to reflect the behavior of trophoblasts that have invaded the maternal bloodstream, are in contact with oxygenated blood and have decreased their invasive phenotype. These phenotypical changes could be related to the modulated expression of HIF-1. Indeed, expression of HIF-1α is high during the first trimester of gestation, decreases after 9 weeks when placental pO_2 levels are believed to increase, and is absent at 11–14 weeks and afterwards [70]. This observation fits with the hypothesis that the placental environment is hypoxic during the first trimester of gestation until invasive CTBs invade the uterine arteries [112–115]. The expression of pVHL, implicated in initiating the degradation of HIF-1α, is highly expressed at sites of column initiation and is increased by hypoxia [116].

Conclusions

A large body of literature is now available to better explain the molecular mechanisms of trophoblastic invasion. However, important questions still remain unanswered, and need further investigation. Understanding trophoblast invasion could also provide insights into the pathogenesis of preeclampsia. These data could constitute the basis for new therapeutic strategies for this disease.

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Signal Transduction in Trophoblast Invasion

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Abstract

During the first trimester of pregnancy, well-differentiated primary cells of the placenta known as trophoblast cells grow in an invasive and destructive fashion similar to malignancies, but limited in space and time. The comparison of trophoblast cells with their malignant counterpart, human choriocarcinoma cells, offers an attractive model to understand the origin or development of malignant growth. Several cytokines and growth factors are known to influence trophoblast migration (e.g. EGF, IGF-2, HGF), proliferation (e.g. leptin, HGF, GM-CSF) and/or invasion (e.g. leukemia inhibitory factor, LIF), each factor utilizing at least one pathway for intracellular signaling in the trophoblast. Two pathways that are crossed especially often mediate the signals of these factors and are simultaneously well established in terms of tumor invasion: the Janus kinase-signal transducers and activators of transcription (Jak-Stat) and receptor-associated tyrosine kinase-mitogen-activated protein kinase (RTK-MAPK) pathways. These two pathways are detrimental for reproduction in general, and in part for placenta development, as a series of knockout experiments demonstrate. Aspects of each pathway are also implicated to be involved in trophoblast invasion, e.g. STAT3 is constitutively activated in invasive first trimester trophoblast cells, and activated ERK is detectable in intermediate trophoblast cells, an invasive phenotype. Interaction at several intersection points between the pathways has been described in several cell systems so that the same would seem to be possible in trophoblast cells. In this review, some of the possible areas of interaction are alluded to.

Introduction: Trophoblast and Tumor as Models for Comparative Investigation of Signal Transduction

Analogies between the fetus and cancer have frequently been made [1–4]. This is mainly because blastocystic cytotrophoblast cells (CTB) and extravillous

trophoblast cells (EVT), often termed intermediate trophoblast, display highly invasive features especially during implantation and the first trimester of pregnancy. Several aspects of malignant and trophoblastic invasion, migration and proliferation are similar, if not identical. The adhesion molecule and protease profile as well as the underlying autocrine/paracrine dialogue involved in attachment and assault and the method of evasion from host (maternal) immune system are comparable. One chief difference, though, between the two situations is spatial and temporal containment. Invasive growth by EVT is restricted to the decidua of first trimester uteri in healthy pregnancies. Pathological invasion and destruction involved in tumor expansion knows no such boundaries. Another difference is that trophoblast cells, being physiological cells, remain well differentiated, whereas malignant cells are transformed, thus exhibiting signs of dysregulation, especially in respect to survival, proliferation and invasion.

This unique comparability makes EVT an attractive model for investigating intracellular regulatory mechanisms of invasion, particularly because information pertaining to the promotion and demotion of invasion during a normal course of pregnancy is deficient on the signal transduction level.

The fact that trophoblast cells are not only able to implant in other organs, as in ectopic pregnancies [5], but also to act as invasive xenogeneic transplants, e.g. murine renal subcapsular space [6], implies that trophoblast cells themselves greatly contribute to the mechanism of invasion.

Numerous cytokines, often secreted by the trophoblast cell themselves, have been described to influence trophoblast migration, proliferation, differentiation and invasion. Naturally, these cytokines exert their effects through use of one or more signal-transducing pathways (assorted selection of cytokines, table 1). Two pathways seem to be frequented more often: the signal transducer and activator of transcription (Stat)- and mitogen-activated protein kinase (MAPK)-mediated ones. A more detailed description follows.

Janus Kinase-Stat Signal-Transducing Pathway

The Pathway

Cytokine binding leads to receptor aggregation, resulting in the juxtaposition of the J*a*nus kinases (Jaks), which are receptor-associated tyrosine kinases named so because of their two symmetrical kinase-like domains, bearing similarity to the two-headed mythological Roman god, Janus [7]. The Jaks are now able to cross-phosphorylate and activate each other, as well as tyrosine residues on cytoplasmic domains of their respective cytokine receptor, thus leaving behind binding points for phosphotyrosine-binding (PTB) domains or any protein processing Src homology 2 (SH2). These

Table 1. Assorted cytokines, their effects on human trophoblast cells and signal transduction

Mediator	Invasion	Proliferation	Migration	Differentiation	Signal transduction	Effect of signal transduction
EGF (epithelial growth factor)	↑ [58]	↑ [59]	↑ [56]	↑ to syncytium [60]	MAPK, PI3K [56]	Migration [56]
CSF-1 (colony-stimulating factor-1)	– [61]	↑ [61]	?	↑ to syncytium [60]	?	?
VEGF (vasoendothelial growth factor)	↓ [62]	↑ [62]	↓ [62]	↑ to syncytium in CTB [63]	MAPK (in Jeg-3) [64]	Probably invasion (MMP ↑) [64]
PIGF (placental growth factor)	↓ [65]	↑ [65]	↓ [65]	– [63]	MAPK [34]	Uterine Vasculogenesis [34]
IGF-2 (insulin-like growth factor-2)	↑ [66]	– [67]	↑ [68]	?	MAPK [68]	Migration [68]
TGF-β (transforming growth factor-β)	↓ [66]	↓ [69]	↓ [66]	↓ to syncytium [60]	Smad3 [70]	?
Decorin	↓ [66]	↓ [71]	↓ [66]	?	For trophoblast? PKB for endothelial cells [72]	?
IGF-1 (insulin growth factor-1)	↑ [73]	↑ [59]	↑ [74]	↑ to syncytium [75]	?	?
GM-CSF (granulocyte macrophage-colony-stimulating factor	?	↑ [76]	?	↑ to syncythium [60]	PKC (in Jeg-3, JAR) [77]	Production of ovine IFN-τ [77]
HGF (hepatocyte growth factor)	↑ [78]	↑ [79]	↑ [80]	↑ to syncytium (in mice) [81]	MAPK, PI3K [82]	Motility [82]

Table 1. (continued)

Mediator	Invasion	Proliferation	Migration	Differentiation	Signal transduction	Effect of signal transduction
LIF (leukemia-inhibitory factor)	↑ [19]	↑ [19]	?	↑ indirectly, to syncytium [83]	gp130 [19]	Invasion, proliferation [19]
TNF-α (tumor necrosis factor-α)	↓ [84]	↓ [85]	↓ [84]	↓ to syncytium [86]	NK-κB [87]	Production of prostaglandins [87]
Leptin	↑ (in mice) [88] ↑ (MMP↑) [89]	↑ (in JAR) [90]	?	↑ to invasive phenotype [89]	Stat (murine oocyte) [91] MAPK in JAR [90] in BeWo [39]	Oocyte maturation [91] Proliferation [39, 90]
IL-10	↓ [92]	?	?	?	?	?
IL-6	↑ [19]	↑ [93]	?	– [86] ↑ (to syncytium [93]	For trophoblast? gp130 for lymphocytes [94]	?
IL-1β	↑ [95]	– [96]	↑ [97]	– [98]	For trophoblast? MAPK, NK-κB (in baboon decidua) [99]	?

include the Stats as well as Ras, phosphatidyinositol 3-kinase (PI3K) and phospholipase C-γ [8].

Upon binding the tyrosine-phosphorylated receptor ligands, the Stats are themselves phosphorylated on specific tyrosine or serine residues by the Jaks, following which the Stats disassociate from the ligand, proceed to form homo- and heterodimers with other phosphorylated Stats and translocate into the nucleus where they bind to specific DNA sequences in the promoter regions of target genes, thus regulating the transcription of these proteins, fulfilling the prophecy implied in their names [9]. Stats also accelerate the transcription of suppressors of cytokine signaling (SOCS), which inhibit the Stat-mediated signal transduction as classical feedback inhibitors [8].

Introducing Stat3

One of the possible regulatory candidates is Stat3. Aberrant activity of phosphorylated, dimerized Stat3 is advocated to be causal for neoplastic cell behavior, e.g. hyperplasia, longevity or invasion, and thus for the malignancy of cells [10]. Indeed, constitutively activated Stat3 has been found in a number of tumors [11].

Stat3 Knockout Model: Relevance for Reproduction?

Evidently, Stat3 has proven to be indispensable for murine pregnancy as it is activated during the early postimplantation period of the mouse and is essential for embryogenesis. Wild-type mouse embryos express Stat3 on the extraembryonic visceral endoderm 7.5 days postcoitum. Concurrent to this, Stat3–/– mice degenerate and die, but can be rescued through substitution with an alternative splice form of Stat3, Stat3β, in which the C-terminal transactivation domain is replaced with a seven-amino acid extension [12–14].

Furthermore, several cytokines that are relevant to reproductive biology, especially those of the interleukin-6 (IL-6)-type family, can activate the Jak-Stat signal-transducing pathway [15]. The IL-6 receptor consists, amongst others, of the signal-transducing subunit gp130 which is common for all receptors of the IL-6-type family and through which the signal is generated [16, 17].

Stats and Trophoblast Invasiveness

Evidence illustrating that the presence of constitutively activated Stat3 correlates positively with the invasiveness of trophoblast phenotypes or their malignant derivates, choriocarcinoma cells, has recently been exposed [18]. Here, it was demonstrated that constitutive activation of Stat3 ceased at the same time as loss of invasive properties of trophoblast cells progressively from first trimester to term. However, highly invasive choriocarcinoma cells constitutively expressed activated Stat3 to a much higher degree than the invasive trophoblast phenotype.

A further study indicated that LIF was able to trigger Stat3 activation in Jeg-3 choriocarcinoma cells, a rather low-invasive cell line compared to other choriocarcinoma cells [19]. The higher activation of Stat3 led to higher proliferation and invasion rates, which seems to be due, at least in part, to the suppressed expression of tissue inhibitor of metalloproteinase 1 (TIMP-1) in conjunction with an increased caspase-4 expression. TIMP-1 is linked to the inhibition of metastasis [20] by inhibiting all metalloproteinases (MMPs), but preferentially binding MMP-9 [21], which is considered crucial for CTB invasion [22]. Caspase-4, formerly coined IL-1β-converting enzyme homologue 2 (ICH-2), enzymatically produces the bioactive form of IL-1β, as its name implies [23]. The significance of IL-1β for invasion can be gathered from table 1.

Another noteworthy fact is that four further cytokines or growth factors (GM-CSF, IGF-2, HGF, and IL-6), propagated to mediate their respective effects through tyrosine phosphorylation of Stat3, were also investigated regarding their capacity to activate Stat3 in Jeg-3 choriocarcinoma cells. LIF was the only cytokine capable of triggering Stat3 activity, while the above-mentioned cytokines were negative in this respect [19]. This suggests that these cytokines use alternative pathways to mediate their effects although they all possess the gp130 signal-transducing subunit. This point will be discussed later.

Furthermore, it should be remembered that Jeg-3 cells are transformed trophoblast cells, and serve solely as a model for trophoblast behavior. It remains to be investigated whether this susceptibility towards LIF with the resulting change in proliferation, invasion and protease expression is a manifestation of their transformed state or whether this is indeed a mechanism that physiological trophoblast utilize as well. One study seems to support the idea that trophoblast cells react in the same manner as choriocarcinoma cells in respect to LIF [24]. Stat3 was functionally knocked down in Jeg-3 choriocarcinoma, first trimester and term trophoblast cells via RNAi (RNA interference) and then assessed for invasion with or without LIF supplementation. In invasion assays with control cells, where no Stat3 was knocked down, invasion was significantly elevated through LIF supplementation for all mentioned cell types including the usually noninvasive term trophoblast cells. As to be expected, choriocarcinoma cells were the most and term trophoblast cells the least respondent to LIF stimulation. Upon Stat3 knockdown, invasive capacity of all cells were clearly reduced irrespective of the presence of LIF [24].

Ras Protein-MAPK Signal-Transducing Pathway

The significance of the Ras-MAPK for cellular programs such as proliferation, differentiation, development, survival, transformation and apoptosis has

been maintained in many papers and reviews in the past. In this report excessive details have been omitted in order to achieve clarity. The following contains an outline of some interesting findings as regards certain aspects of reproductive or tumor immunology and biology.

The Pathway

Upon activation of receptor tyrosine kinases (RTK) through specific cytokine-receptor interactions, RTK phosphorylates tyrosine residues on its inner receptor ligands in a fashion reminiscent of the Jaks, as mentioned above. These binding points attract several signaling proteins, such as the Grb-2 (growth-factor-receptor-bound) adaptor protein or SH2-domain-containing tyrosine phosphatases (SHP2), which simultaneously binds to further proteins that ultimately activate monomeric GTPases termed Ras. This sets off a chain reaction resulting finally in the activation of MAPK through consecutive activation of first MAPK kinase kinase (MAPKKK) and then MAPK kinase (MAPKK). The activated MAPK can enter the nucleus and phosphorylate one or more components of a gene regulatory complex (fig. 1), which activates the transcription of a set of immediate early genes (named so because they are so very rapidly turned on in response to extracellular signals). The resulting change in gene expression leads to changes in cell behavior modulating protein activity [as reviewed in 25].

Introducing MAPK

MAPKs are subdivided into four main groups: the classical MAPKs, c-Jun N-terminal kinases (JNKs, often called 'junks', but also referred to as stress-activated protein kinase, SAPK), p38s, and atypical MAPKs. Numerous isoforms of MAPKs, MAPKKs, MAPKKKs and Ras have been identified in mammalian cells (table 2) [26]. Notably, MKK4 has been recognized as a putative tumor suppressor gene in human solid tumors of the breast, prostate and pancreas [27].

MAPK and Reproduction

MAPK and the Oocyte. Since MAPK is pivotal in the cell cycle regulation, it is no surprise that MAPK is also involved in oocyte maturation. Oocytes express the Mos protein during meiotic maturation from the G2 to M phase. MAPK activation through the Mos protein seems to be necessary for activation and stabilization of the M phase-promoting factor, a protein considered to be the master of cell cycle switch. However, Mos protein expression in ectopic, somatic cells results in neoplastic transformation (or uncontrolled G1/S transitions) of these cells [28, 29].

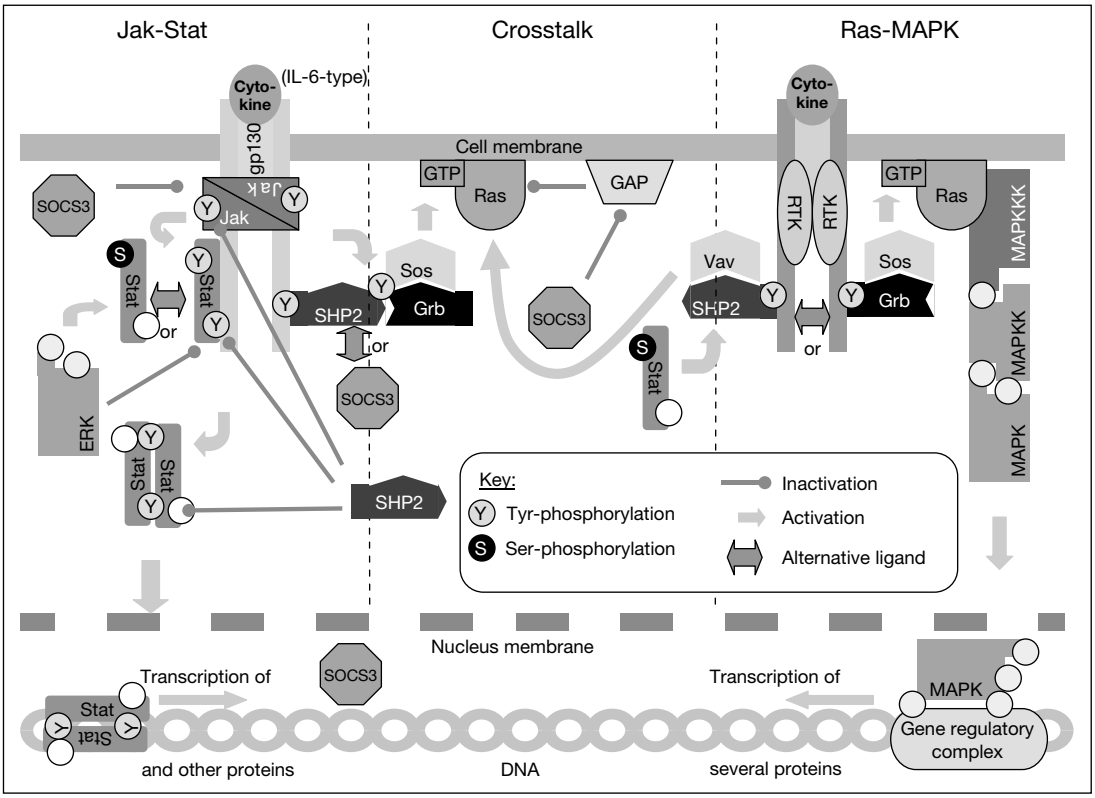

Fig. 1. Simplified animation of the interaction between Jak/Stat and Ras-MAPK pathways as interpreted from the current literature. Cytokine activation of the Jak/Stat pathway leads to transcription of a variety of proteins including SOCS. These regulate the intensity of the signal by binding to Jaks. Activation of the Ras-MAPK pathway occurs via different mechanisms: phosphorylated tyrosine residues on the inner receptor ligands attract Grb-2 or SHP2, which simultaneously bind to further proteins (Vav or Sos, respectively) that ultimately activate Ras. Alternatively, Jak1-phosphorylated SHP2 may act as an adapter by linking Grb-2, which in turn links Sos and activates Ras. Simultaneously, it competes with Stat3 for the tyrosine phosphorylated binding site on the inner receptor ligand. The cross talk between Jak/Stat and Ras-MAPK systems is partly regulated by SOCS, which can compete with SHP2, but which can also block GAP, a deactivator of Ras. On the other hand, ERK, a kinase of the MAPK system, induces Stat serine phosphorylation which may compete with tyrosine phosphorylated Stats. It can also promote the activation of SHP2.

MAPK Knockout Models. Knockout models of various MAPK signal-transducing components in the mouse have led to the perception that MAPK signaling is also important in the development of the placenta. MEKK3, p38α and ERK5–/– mice all display defects in angiogenesis and placental formation

Table 2. Major MAPK cascades in mammalian cells [28]

Activators	Growth factors, cytokines	Growth factors, cytokines, stress, TGF-β	Growth factors, cytokines, stress, ceramides
	↓	↓	↓
MAPKKK	Raf	MEKK 1-4, MLKs, ASK, TAK1	MEKK 1/4, MLKs, ALS, TAK1
	↓	↓	↓
MAPKK	MEK 1/2 (MKK 1/2)	MKK 3/6, MKK4	MKK 4/7
	↓	↓	↓
MAPK	ERK 1/2	P38	JNK/SAPK
	↓	↓	↓
Substrates	90^{RSK}, MNK 1/2, Ets, Elk1, Myc, Stat 1/3, ER	Hsp 27, PLA2, MNK 1/2, APKA2, Myc, MSK-1, Elk1, ATF-2, Stat1	c-jun, ATF2, Elk1, DPC4, p53, NFAT4
	↓	↓	↓
Cell responses	Proliferation, differentiation, development	Proliferation, differentiation, development, inflammation, apoptosis, stress response	Proliferation, differentiation, apoptosis

that lead to embryonic lethality [30–33]. The detrimental placental deviations due to (lack of) the above-mentioned factors point to anomalies especially in placental vasculogenesis. Particularly the labyrinth layer seemed to be greatly reduced in all three investigations; however, p38α mutants also exhibited a significant alleviation of spongiotrophoblast (the murine correlate to column CTB), which seemed to be due to a higher rate of apoptosis. Development of trophoblast giant cells (the murine correlate to invasive CTB) were apparently not affected. This suggests an independent requirement for p38α in diploid trophoblast development. The observation that p38 MAPK activation is perceptible in human syncytiotrophoblast in response to PIGF in vitro could support this theory [34].

MAPK and the Endometrium. Trophoblast cells must first penetrate the luminal epithelium of the endometrium before invasion into the endometrial stroma. A line of reasoning valuable to trophoblast invasion is the aspect that luminal endometrial epithelial cells (EEC) and decidualized endometrium play a role in regulating trophoblast invasion. BeWo choriocarcinoma cells that were cocultured with EEC attached to EEC prior to expansion and invasion, which resulted in a significantly higher rate of apoptosis in EEC. As a consequence thereof, p38 MAPK were activated in EEC. On the other hand, the inhibition of p38 MAPK induced the inhibition of both EEC apoptosis as well as trophoblast invasion [35]. Moreover, endometrial stromal cells respond toward IL-1β with the stimulation of proinflammatory cytokines and an upregulation of p38 MAPK. Inhibition of p38 MAPK and decidualization of EEC through progesterone diminished this IL-1β-induced response, indicating that decidualization leads to a diminished proinflammatory response to IL-1β of EEC through attenuation of IL-1β-induced p38 MAPK activation [36]. This could be of possible relevance for the control of trophoblast invasion.

MAPK in Trophoblast Cells. The information thus far would seem to suggest that MAPK is not involved in trophoblast invasion, or if so, then only indirectly. However, a recent study of expression and activation of MAPK and ERK patterns in human villous trophoblast cells, mostly through immunohistochemistry and in situ hybridization, suggests the contrary. It was demonstrated that villous syncytiotrophoblast cells were negative for total and activated ERKs throughout pregnancy [37]. Villous CTB cells were positive for total ERK throughout pregnancy, but activation of this could be seen only from the 1st to the 12th week of gestation. This activation correlated with c-met (HGF receptor) expression and rate of proliferation. This information agrees well with past studies pointing to the role of HGF in trophoblast proliferation and MAPK in proliferation processes in general. Surprising, though, was the piece of evidence showing that intermediate trophoblast cells (invasive EVT cells) were positive for total and activated ERKs throughout pregnancy. It should be noted that these

cells are not proliferative [38], so that the possibility of linking MAPK activation to intermediate trophoblast invasion is principally possible.

The fact that VEGF could upregulate the expression of MMPs through MAPK activation would reinforce this thought.

Possible Cross Talk?

A Few Examples: Leptin Receptor and gp130

As mentioned earlier, it appears that certain cytokines generate their effects through certain pathways in certain cells, although they feature the capability to transduce their signals through other pathways in other cells. Leptin, for example, is a substance which is able to modulate trophoblast activity, and for which leptin receptors exist on trophoblast cells. The leptin receptor contains a gp130 receptor subunit through which it could potentially transduce its signal to Jak and Stat, but at least in BeWo choriocarcinoma cells, leptin could not enhance Jak2, Stat1 or Stat3 phosphorylation, although Jak2 was constitutively activated. In contrast to this, leptin stimulated cell proliferation and the c-fos gene, an immediate early gene, through the phosphorylation of p42-MAPK [39].

Furthermore, one group generated mice with a COOH-terminal gp130$^{\Delta Stat}$ 'knockin' mutation, which deleted all Stat-binding sites, in order to define Stat-dependent responses. As in LIF–/– mice, in gp130$^{\Delta Stat}$ mice blastocysts also failed to implant, and gp130$^{\Delta Stat}$ mice generally phenocopied LIF or IL-6-deficient mice, with the exception of gastrointestinal ulceration and severe joint disease. It was shown here that this mitogenic hyperresponsiveness of synovial cells in response to the cytokines mentioned was due to sustained gp130-mediated SHP2/Ras/ERK activation, which would normally have been limited through induction of suppressor of cytokine signaling 1 (SOCS1), usually induced through Stat signaling [40].

MAPK-Stat Interactions

These findings indicate that a balance exists between the several transducing pathways and that the regulation thereof takes place through communication between them. The following encompasses an attempt to illustrate recent advances made in defining aspects of signal modulation between the Stat and MAPK systems.

As alluded to earlier, activation of IL-6-type cytokine receptors also leads to MAPK activation, sometimes with the effect of preventing Stat activation. There appear to be a few modes in which this can develop.

Direct Mechanism. One study exposes a direct mechanism of MAPK intervention in the Stat pathway [41]. IL-1 was shown to directly and specifically

inhibit IL-6-induced Jak-Stat signaling through activation of the p38 system independent of protein synthesis (e.g. SOCS).

Tyr759: A Common Binding Site. The Stat-binding site Tyr^{759} on gp130 and Tyr^{974} on LIF receptor of certain cells attracts SHP2, which is then phosphorylated by Jak1. SHP2 may now act as an adapter by linking Grb-2, which in turn links Sos (Son of sevenless) and activates Ras (fig. 1). Ras activates the MAPK cascade, as mentioned above. This can be exemplified when Tyr^{759} is mutated or substituted. Here, the MAPK cascade is downregulated, while LIF and IL-6 signaling is increased. However, SHP2 may also act as a phosphatase on Tyr^{759} or any other tyrosine-phosphorylated signaling components, resulting in the deactivation of gp130 and the Jak-Stat signal-transducing pathway (fig. 1). This is made clear by the fact that overexpression of dominant-negative SHP2 mutants also leads to enhanced receptor, Jak, Stat and SHP2 phosphorylation [42].

Phosphorylation of Stat3. Stats can be phosphorylated at several sites in order to be activated. Tyr^{705} is the best-characterized binding site of Stat3 and leads to the effects described earlier (dimerization and activation of transcription). Another possibility for phosphorylation is Ser^{727} (fig. 1), which occurs at a slower rate than at Tyr^{705} [43]. Unfortunately, to date there is no clear picture of exact cause and effect mechanisms [as reviewed in 42, 44]. The literature mostly indicates an increase in the Stat-induced transcription of target genes upon Ser^{727} phosphorylation of Stats. However, phosphorylation of Ser^{727} on Stat3 can decrease Stat3-dependent transcriptional responses. Indeed, Ser^{727} phosphorylation of Stat3 does not enhance tyrosine phosphorylation of Stat1 or 3, but instead decreases it in some cases. Furthermore, phosphorylation of Ser^{727} leads to activation of the MAPK system through Vav, Rac, MKK4 and PKC. PKC associates with Stat3 in the nucleus and inhibits Stat3 DNA-binding and transcription activity [45, 46]. Reciprocally, activation of ERK, which has been linked to the negative regulation of Stat3, leads to phosphorylation of Ser^{727} on Stat3 (fig. 1). HGF stimulated proliferation of human aortic endothelial cells through the ERK-phospho-Ser^{727}-Stat3 pathway [47]. ERK also causes the inhibition of tyrosine phosphorylation, thus inhibiting Stat3-mediated gene transcription [44].

In summary, it can be established that MAPK plays an important role in the phosphorylation of Ser^{727} residues, but caution is necessary in that serine phosphorylation can occur through several different signal transduction pathways and does not evoke the same effect in every cell.

MAPK and SOCS. Another regulation junction between the two systems can be distinguished in SOCS. On the one hand, tyrosine phosphorylated SOCS (induced through IL-2 or EGF) binds and inactivates the GTPase-activating protein (GAP), which normally inactivates Ras [48]. This ultimately prolongs MAPK activation (fig. 1). Additionally, it could be demonstrated that IFN-γ

stimulation of macrophages infected with the Listeria bacteria responded with a p38 MAPK-mediated Ser^{727} phosphorylation of Stat1 and a higher expression of SOCS3 [49].

Altogether this would indicate a synergistic mechanism between the MAPK pathway and SOCS, but an earlier investigation by Ernst et al. [40] stipulates the contrary as Stat-induced expression of SOCS1 was hypothesized to inhibit MAPK signaling. The possibility that SOCS can inhibit both pathways remains to be explored. SOCS binds with a higher affinity to Tyr^{759} than SHP2 [50], which would suggest just such a mechanism (fig. 1). In addition, overexpression of SOCS3 in human melanoma cell lines could completely abolish activation of both Jak-Stat and Ras-MAPK signaling [51].

Mammalian Target of Rapamycin-MAPK-Stat Interactions

Mammalian target of rapamycin (mTOR), belonging to the phosphoinositide kinase-related kinase family, is involved in cell growth and the cell cycle, control of the cytoskeleton and nutrient transport, protein and RNA stability and transcription and translation [52]. mTOR regulates the rate of protein translation in response to growth factor or mitogenic signals, allowing progression from the G1 to S phase of the cell cycle [53]. In a mouse model it could be demonstrated that disruption of the mTOR gene was implicated with a limited level of trophoblast outgrowth in vitro and postimplantational lethality of homozygotes [54]. Rapamycin treatment of mouse embryos also inhibits trophoblast outgrowth [54]. A deletion of 6 amino acids in the C-terminal part essential for kinase activity of mTOR demonstrated a reduced cell size and a proliferation arrest in early mouse embryos and embryonic stem cells [55]. It has been suggested that mTOR/p70S6K1 is involved in MAP kinase-mediated, but not PI3K-mediated, trophoblast migration in response to EGF since it could be shown that rapamycin inhibited cell migration and p70S6K1 phosphorylation [56]. In neuroblastoma cells mTOR phosphorylates Stat3 at Ser^{727} by the ciliary neurotrophic factor, which leads to a maximal transcriptional activation together with the Tyr^{705} phosphorylation by Jak kinases [57].

In summary, it is suggested that mTOR, MAPK and Stat pathways interact and potentially can regulate or modulate each other, but depending on which cell and which extracellular signal is being observed, different mechanisms with both synergistic and antagonistic effects are discernable.

Conclusions

Signal transduction in trophoblast invasion, especially in comparison to neoplastic invasion, is an area of reproduction or tumor biology that encourages

further investigation. First, because of the distinct yet similar situations of both types of growths and second, because data, especially concerning trophoblast invasion, is lacking. Particularly two pathways, the Stat- and the MAPK-mediated ones, have been distinguished as being involved in this process. Several studies suggest that these two pathways intersect at the point of modulation, but exact theories are not easily reconciled and instead invite further exploration. Understanding the signal transduction mechanisms of (trophoblast) invasion and their cross talk could assist in clarifying pathologies, such as multiple implantation failure or development of cancer, but also provides impetus for new ideas in respect to therapy.

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