In Pursuit of Genetic Factors for Recurrent Pregnancy Loss

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1. Recurrent Pregnancy Loss (RPL)

The maintenance of human pregnancy involves immunological, metabolic, vascular and endocrine regulatory processes that are genetically controlled. Abnormal regulation of these processes may be involved in recurrent pregnancy loss (RPL), which is defined as the loss of three or more consecutive pregnancies before the 20th of gestation, and which affects 2~5% of couples trying to conceive. However, in a significant proportion of cases, the underlying cause of this problem is largely ill defined other than for chromosomal abnormalities. Epidemiological surveys indicate that the etiology for 24~60% all cases of RPL remains unresolved. Previous investigations have focused on parental chromosomal abnormalities, congenital malformations of the uterus, endocrine abnormalities, immunological disorders, and homeostatic and metabolic abnormalities. Recently, immunological studies have been carried out more actively than before. It has been reported that immunological factors involved in RPL are cytokine (Th1/Th2) balance, placental protein 14 (PP14), annexin II, mannan binding lectin (MBL), mucin 1 (MUC1), leukemia inhibitory factor (LIF), indoleamine 2, 3 - deoxigenase (IDO), cluster designation 95 (CD95), and suppressor macrophage. Since the conceptus contains paternal gene products and its immune differentiating antigen, it is possible that the maternal immune system recognizes these gene products as immunologically foreign, resulting in rejection. However, the incidence of this is most likely rare owing to the fact that uterine deciduas secrete soluble proteins capable of inhibiting cell-mediated immune responses, potentially protecting the conceptus from maternal immune rejection during pregnancy.

Abnormal expression of several endometrial proteins including PP14 (also known as the pregnancy-associated endometrial - 2 globulin or glycodelin), CA125, and MUC1 (mucin 1) for RPL has been described. Since they are secreted by the endometrial glandular epithelium, the concentration of these proteins can be detected by endometrial flushing. PP14 is secreted by the ovary and the endometrium under the influence of progesterone and may serve as an immunosuppressor potentially by protecting the feto-maternal tissues. The concentration of PP14 has been reported to be significantly lower in endometrial and uterine flushings from RPL patients than in those from normal fertile controls. CA125 is also produced by both the ovary and the endometrium and has been reported that it is found less in the endometrial flushings from RPL patients than in those from the normal fertile controls. In addition to PP14 and CA125, MUC1 has also been reported to be lower in uterine flushings from women suffering RPL than in normal fertile controls. Therefore, it is expected that there are genes expressed to aberrant extent in RPL patients. This led us to investigate gene expression levels in normal pregnancy and in

pregnancy resulting in or from RPL, using the molecular cloning and the expression pattern of genes in chorionic villi via cDNA subtractive hybridization analysis.

2. Subtractive Hybridization and RT-PCR Analyses

Subtractive hybridization is a powerful technique that enables researchers to compare two populations of mRNA and obtain clones of genes that are expressed in one population but not in the other. Although there are several different methods, the basic theory behind subtraction is simple. First, both mRNA populations are converted into cDNA: we refer to the cDNA that contains specific (differentially expressed) transcripts as tester, and the reference cDNA as driver. Tester and driver cDNAs are hybridized, and the hybrid sequences are then removed. Consequently, the remaining unhybridized cDNAs represent genes that are expressed in the tester, but are absent from the driver mRNA. Although traditional subtractive hybridization methods have been successful in some cases, they require several rounds of hybridization and are not well suited for the identification of rare messages. This is a unique method based on selective amplification of differentially expressed sequences, which overcomes technical limitations of traditional subtraction methods. The PCR-based subtractive hybridization analysis has been used for investigating a variety of subjects in living organisms. We performed subtractive hybridization and RT-PCR analyses of cDNAs from the chorionic villi of normal and RPL patients, and identified at least eight genes, including two as yet unidentified genes, whose expression is significantly reduced in RPL patients. Four of them have been reported to regulate immunosuppression, angiogenesis, or embryo attachment.

1) Immunosuppression-related Genes

Recent studies have been focusing on immunological maternal-fetal reaction that may cause RPL. Since a half of the fetal genome derives from the father, the fetus synthesizes antigens considered to be foreign by the maternal immune system. How succeed pregnancy against the maternal immune system is not well-specified, but the most reliable hypothesis is involved in an immune barrier by the placenta. One of immunosuppression-related molecules is glycodelin. It has been called by various names; placental protein 14 (PP14), chorionic 2-microglobulin (CAG-2), progesterone-associated endometrial protein (PEP) and pregnancy-associated 2-microglobulin (2-PEG). PP14 has been isolated from human placenta and is expressed in other normal tissues including the epithelium of the fallopian tube, ovarian surface epithelium, breast tissue, sweat glands, and bone marrow aspirates. One of biological functions for PP14 is to inhibit early events in the T-cell receptor signaling pathway. Previous studies showed that PP14 inhibits phytohemaglutinin-induced lymphocyte proliferation and IL-2 synthesis. PP14 also inhibits the IL-2-induced activity of natural killer cells in culture. Taken all together, PP14 has important roles as a possible immunosuppressive molecule capable of successive pregnancy and growth of the fetus. In addition, PP14 is known as an inducer for apoptosis in T cells and is involved in carcinoma mechanisms.

Mucin proteins generally contain a series of tandem repeat domain enriched in serine, threonine, and proline residues. Many human mucin genes excluding MUC5B are polymorphic. 10 mucin genes have been discovered so far and are nomenclatured followed by a number reflecting the order in which the particular mucin gene was

discovered (MUC1, MUC2, MUC3, and etc.). Mucins have various functions; protection against bacterial infections, protection of proteins and cells from proteolysis, inhibition of cell attachment, promotion of cell attachment, and inhibition of immune cell function. It has been found that MUC1 is a cell-surface and secretory molecule of endometrial epithelium and shows the highest expression in the mid-luteal phase. Previous studies demonstrated that RPL patients showed low MUC1 protein concentration relative to normal pregnancy controls analyzed in uterine flushing.

2) Angiogenesis-related Genes

Angiogenesis, the formation of new blood vessels from pre-existing blood vessels, is a fundamental process occurring during embryonic development, the reproductive cycle, wound healing, and cancer development. Angiogenesis is stimulated by factors such as vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF). An early response to angiogenesis-stimulating factors is the degradation of endothelial cell basement membrane by proteases, for example, by members of the matrix metalloproteinase (MMP) family. MMPs degrade collagen and other extracellular matrix components, disrupting the basement membrane barrier, enabling endothelial cells to migrate from pre-existing vessels towards angiogenesis stimuli and to proliferate. Vascular cell-adhesion molecules contribute to endothelial cell migration by mediating cell-extracellular matrix interaction. An important mediator is the integrin, a receptor for Arg-Gly-Asp (RGD)-containing proteins such as fibronectin which is expressed at low levels in quiescent blood vessels. Integrin expression is induced following exposure to angiogenesis-stimulating factors, preferentially on the surface of endothelial cells in newly forming capillaries. During gestation, angiogenesis occurs extensively in placenta and villi to provide oxygen and nutrition for fetus. Deficiency of this process gives rise to abortion or abnormal growth of fetus, which lead to a marked increase in uterine and umbilical blood flows. It has been preciously reported that reduced placental vascular development is associated with early embryonic mortality. However, there is little information for the importance of normal angiogenesis at the molecular level for RPL.

MMPs are involved in basement membrane disruption by T lymphocytes. MMP family members have been classified according to their substrate specificity; gelatinases (MMP-2, MMP-9), stromelysins, and collagenases (MMP-1). It has been found that gelatinases degrade basement membrane components including collagen types IV and V, fibronectin, entactin, and elastin. Previous reports showed that MMP-2 and MMP-9 are expressed under the influence of T lymphocytes. However, the relation between MMP expression and activation by T lymphocytes has not been well-clarified. Fibronectin is a component of epithelial and endothelial cells that synthesize extracellular matrix structure. Fibronectin has two forms in human; one is a soluble dimeric plasma protein and the other is an insoluble multimeric matrix protein. Both forms are similar in many biological functions, including platelet adhesion, endothelial cell integrity, and cell migration during blood vessel repair. In addition, the insoluble fibronectin in the matrix plays a role in the mediation of attachment and migration during embryonic development and tissue rearrangement. It has been demonstrated that fibronectin upregulates MMP-2 and MMP-9 in human T cell mediated adhesion and migration, which are associated with wound healing in lymphocytes. However, it has been shown that the adhesion of T cells to fibronectin increases the frequency of RPL. Fibronectin also protects against TNF-induced toxicity in human trophoblast. Thus, this molecule seems to

have various functions in immune response and further investigations have to be done to better understand its cellular functions.

3) Other Genes

hCG is a luteotrophic hormone that maintains the corpus luteum and is detected in the maternal blood during the second week of pregnancy and its concentration reaches a peak during the 7 th to 12 th weeks. hCG stimulates placental steroid synthesis and the growth of the fetal adrenal gland. In addition, hCG is involved in immunological reactivity of the maternal tissues, altered by immunosuppressive action via maternal leukocytes in the region of the invading trophoblast. The human globin genes are composed of multigene locus. Each globin gene (ε, $^{G}\gamma$, $^{A}\gamma$, δ, and β) is expressed in different developmental stages. The switch from fetal (Hb F) to adult (Hb A) hemoglobin takes place for a life cycle. For instance, embryonic ε-globin gene is expressed until the 5 th week of gestation in the yolk sac. After the ε-globin gene was expressed, the first change is the conversion of gene expression to fetal globin ($^{G}\gamma$, $^{A}\gamma$) genes, taking place until the birth. After the expression of fetal globin genes ($^{G}\gamma$, $^{A}\gamma$), the subsequent change occurs with the expression of adult β-globin gene. However, the expression of fetal γ-globin gene continues throughout the life cycle, with much less extent (1%) than that of adult β-globin gene (98%). Disorders resulting from abnormal expression of these genes are β-thalassemia and sickle cell disease (SCD). Recent studies showed that increase in (γ) globin gene expression leads to the improvement of symptom, resulting in reduced dyserythropoieses and transfusion requirements because of its binding with the β-chains in the β-thalassemia. It has been reported that in SCD, Hb F has important roles as an inhibitor for the polymerization of deoxyhemoglobin S. A number of mechanistic problems for hemoglobin-related diseases are left to be solved. At the protein expression level, several proteins, including PP14 and hCG, were detected more in flushings from the uterine of normal patients than in that of RPL patients. It has been suggested that PP14 functions as an immunosuppressor for the protection of the maternal-fetal tissues from potentially hostile maternal immune system and PP14 concentration in uterine is an adequate indicator for diagnosing whether the pregnancy will succeed or not due to their different level of protein expression in both normal and RPL patients. hCG always keeps in the concentration of 65,600 IU/L, which lasts until the 9 th week of pregnancy. However, it shows remarkably subnormal concentration during the first trimester in the RPL patients, suggesting that hCG is also a useful marker for the diagnosis of the early pregnancy failure. Functions for some of these genes are involved in cellular processes for T lymphocytes and cytokine functions for immune responses.

It is known that their functions are also related to each other. Th1 cytokines, such as gamma-interferon (IFN-γ) and tumor necrosis factor (TNF-α), may give embryotoxic effects. Approximately 25% of women with a history of RPL has been shown the elevation of immune response to trophoblast, increased proliferation of inflammatory cells, and preferential secretion of embryotoxic Th1 cytokines. TNF-α is produced from activated macrophages and its cytotoxic effects appear in collaboration with IFN-γ. In addition, TNF-α-mediated signal transduction pathway results in various effects including apoptosis, cell proliferation, and cell differentiation. On the contrary, it has been demonstrated that the fibronectin plays a role in blocking cytotoxic effects of TNF-α in cultured human trophoblast cells. It has been shown that MMP-2 and MMP-9 are produced in T lymphocytes and both are induced by IL-2. IL-2 or phobol-ester upregulates the expression of MMP-9, whereas the expression of MMP-2

depends on the duration of IL-2 exposure. Fibronectin not only induces MMP-2 and MMP-9 expression by T-lymphocytes, but promotes MMP-2 activation.

3. Telomerase and Apoptosis

Telomeres and telomerase catalytic subunit (hTERT) are involved in the regulation of cellular life span through stabilization of chromosomal ends preventing chromosome degradation, end-to-end fusion, rearrangement, and loss. Telomeric length is a good indicator for the replicative capacity of a cell, and activation of hTERT for synthesis of telomeric DNA is required to overcome cellular senescence and to attain immortality. High telomerase activity has been described in germ line cells, chorionic villous cells, cancers and immortalized cell lines. In a preliminary study, we have observed that telomerase activity was suppressed in chorionic villi tissues obtained from RPL. This suggests that telomerase activity directly linked to the hTERT isoforms transcribed in the chorionic tissues and is an essential factor for maintaining early pregnancy.

Apoptosis, or programmed cell death, is responsible for many normal developmental processes. It has been demonstrated that it is a crucial process during embryonic development including the reorganization of the adult organism and the surveillance of the cell cycle. Abnormalities in cell death control can contribute to a variety of diseases, including cancer, autoimmunity, and degenerative disorders. It is evident that a balance between cell death and proliferation play a critical role in the maintenance of normal tissue homeostasis. Signaling pathways for apoptosis converge on a common machinery of cell destruction that is activated by a family of cysteine proteases that cleave proteins at aspartate residues (caspases), members of the tumor necrosis factor (TNF) receptor (TNF-R) superfamily, and members of the Bcl-2 family proteins. This can be particularly important for the successful development of human pregnancy. It has been demonstrated that apoptosis in normal pregnancy is very critical. Apoptosis within the maternal deciduas seems to play an important role in the establishment of immune system for the pregnant uterus to protect fetal cells from killing by maternal cells. However, it has not been demonstrated how programmed cell death is involved in normal development of the fetal chorionic villi at the molecular level. Our study revealed that the apoptosis-related genes including caspases 3, 6, 7, 8, 9, 10, 12, Bad, Bax, Fas, and FasL were expressed higher in chorionic villi from RPL patients than normal controls. This suggests that the abnormal expression of apoptosis-related genes is one of primary reasons to be involved in RPL.

4. Summary

In order to keep the normal pregnancy, a number of gene products are required at the feto-maternal interface. We have isolated approximately 30 genes, involved in keeping the normal pregnancy, via subtractive hybridization and RT-PCR analyses of cDNAs from the chorionic villi of normal and RPL patients. Characterizing their functions will help us to understand the process of establishing and maintaining pregnancy. In addition, more detailed studies of their expression in normal and RPL patients are required to evaluate their clinical relevance. Further identification of genes aberrantly expressed in RPL patients will help the prognosis of the pregnancy,

identifying pregnancies with a high risk of miscarriage and enabling management of those pregnancies.

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