懷孕時母體的免疫調適與胚胎著床的關係(3/3)
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INTRODUCTION

The fetal genome is half-paternal and half-maternal. According to transplantation immunology, it is logical to postulate that the maternal immune system should reject it during pregnancy, but this generally dose not occur. Although it is still an open question how the conceptus avoids immune rejection, it is now generally accepted that the maternal adaptive immune system is at least aware that the fetus and placenta exist, as certified by alloantigen-specific alterations in maternal T cell phenotypes during pregnancy.

Interleukin 2 (IL-2) is a potent immunomodulator, whose major function is to activate various cells of the immune system, including helper T cells, cytotoxic T cells, NK cells, B cells and macrophages. It mediates its biological effects through binding to its corresponding cell surface receptors. There are three recognized IL2 receptors (IL-2R), composed from up to three glycopeptide subunits: a 55 kDa α-chain (CD25), a 75 kDa β-chain (CD122) and 64 kDa γ_c-chain (CD132). In the peripheral blood, less than 5% of normal circulating mononuclear cells express the IL-2Rα and, if do, only at very low level. The high-affinity IL-2R, composed of all three subunits, is not expressed on normal or unstimulated lymphocytes, but it is rapidly transcribed and expressed on activated T cells. Until recently the IL-2 pathway was considered as a necessary components of adaptive immune responses to transplanted tissue. Blockade of either the cytokine or the IL-2R prolongs heterotopic allograft survival in rats and mice.

In our previous study, we demonstrated that the levels of CD25 on $CD4^+$ and $CD8^+$ T lymphocytes were decreased during pregnancy, although the levels of CD69 and HLA-DR were markedly increased. Because the IL-2/IL-2R system plays a crucial role in the generation of cytotoxic lymphocytes, the decreased expression of CD25 must be related with the tolerance to the fetoplacental graft. Now we further test whether the reduced IL-2Rα levels on T cell surface are due to decreased IL-2Rα gene expression or the cleavage of CD25 molecules?

MATERIALS AND METHODS

Subjects

Twelve patients (aged 43.9±3.4 years) who received hysterectomies for benign reasons of nonendometrial pathology (mainly myoma uteri) and eighteen pregnant women (aged 33.0±5.2 years) who had elective abortions of normal pregnancies between 6 and 10 weeks of gestational age due to multiparity, were enrolled in the study with informed consent and under the approval of the investigation review board of our hospital. These women had regular menstruation before hysterectomy. The pathologic results of the hysterectomy specimens showed no evidence of infection or inflammation. All of them were in the luteal phase according to the last menstrual period, levels of progesterone, and histological dating. The two groups were matched by the number of previous pregnancies and body mass index.

Specimens

Endometrial tissues and venous blood obtained from hysterectomy patients were collected on the day of operation. Endometrial tissue was cut with a surgical knife immediately after the hysterectomy, minced with a scalpel, suspended in RPMI-1640 medium, and pressed gently through a 380-μm and then a 45.7-μm sieve

as described previously. Decidual tissue, fetal chorionic villi and peripheral blood samples were taken from each pregnant woman at the time of abortion. In order to minimize contamination by blood, the decidual tissue was macroscopically separated from the chorionic villi, washed twice with Hank's balanced salt solution (HBSS: 1 g/L D-glucose, 0.35 g/L sodium bicarbonate, phenol red), cut into small pieces, washed twice again, and passed through a 1.9-mm mesh to remove the residual blood without enzymatic treatment. These samples were then filtered through a 45.7-μm stainless steel mesh to remove tissue debris. The filtered solution was layered over a Ficoll-Paque PLUS gradient and centrifuged for 45 minutes at 400*g*. An enriched cell suspension was collected at the interface and then washed twice with RPMI-1640 medium. The recovered mononuclear cells were checked for viability with trypan blue and counted. Peripheral blood mononuclear cells (PBMCs) were also isolated by Ficoll-Paque PLUS sedimentation. Villous tissue was carefully examined under a dissecting microscope to ensure absence of all maternal tissue and was minced into small pieces. Then the tissue (1.5 gm) was digested in a plastic tube with 6 mL HBSS (Ca^{2+}- and Mg^{2+}-free) containing 0.25% trypsin. The tube was vigorously shaken at 37°C water bath for 30 minutes and was spun at 1000*g* for 10 minutes. The supernatant was aspirated and the large pellet was resuspended in 6 mL RPMI-1640. The cytotrophoblast cells were separated out using a discontinuous Percoll density gradient and were collected from cell banding between 35% and 55%. Thereafter the cells were transferred to a plastic culture dish filled with culture medium (RPMI-1640 supplemented with 100IU/mL Penicillin, 100μg/mL Streptomycin, and 10% fetal calf serum). The medium was changed daily and coculture procedure was performed when the cells were almost confluent.

Autologous Coculture Assay

PBMCs were fully activated with 10 μg/mL of PHA in a 37°C, 5% CO_2-humidified incubator for 24 hours. The activation and coculture procedure outlined below were performed in 12mm × 75mm capped polystyrene test tubes. In the experimental group, 5×10^5 activated T cells were cocultured with 1×10^6 autologous villous cells (1:2) in 3ml of culture medium. In the control group, only 5×10^5 activated T cells were cultured in 3ml of culture medium. The immunophenotyping of activated T cells in both groups was analyzed by flow cytometry on days 1, 3, and 5 of cultures.

Quantitation of CD25 mRNA by Real-Time PCR

Each of the samples subjected to TaqMan PCR analysis was first homogenized, and the total RNA was extracted with Trizol (Gibco-BRL, Invitrogen Co., CA, USA) and the cDNA was synthesized by RT reaction according to the manufacturer's instructions. The PCR reaction was performed in a 50-μL volume of the reaction solution containing 1X TaqMan Universal PCR Master Mix (including AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer components), 1X target or control primers and probe, and 10ng-1μg cDNA sample.

The relative quantitation of gene expression of the IL-2Rα is assessed with TaqMan PCR method. The method is based on the use of fluoregenic probes that anneals to the targeted gene sequence of interest between the forward and reverse primer sites. It exploits the 5' nuclease activity of the recombinant DNA polymerase (AmpliTaq Gold DNA Polymerase) to cleave a TaqMan probe during PCR extension. Each intact probe contains a fluorescent dye reporter at the 5' end and a quencher dye at the 3' end, which inhibits the reporter emission by quenching the energy emission. During the amplification reaction, the 5' nuclease activity of the DNA polymerase cleaves the probe between the reporter and the quencher. This separates the

reporter dye from the quencher dye, generating an increase in the reporter dye's fluorescence intensity.

Once separated from the quencher, the report dye emits its characteristic fluorescence, which can be detected in real-time by monitoring fluorescent energy with the ABI PRISM 7700 Sequence Detector System (Perkin-Elmer Applied Biosystems, CA, USA). This increase in fluorescence is proportional to the concentration of the target sequence in the initial samples. To measure sample of RNA levels, it is necessary to determine the threshold cycle (C_T value), which represents the PCR cycle at which a statistically significant increase (above a baseline signal) in reporter florescence energy is first detected. The relationship between the C_T value and logarithm of the starting copy number of CD25 cDNA is linear under optimum conditions. The more is initially starting copy number of cDNA, the less is C_T value.

The oligonucleotide primers and TaqMan probe for IL-2Rα are provided by using TaqMan Pre-Developed Assay Reagents (PDARs). TaqMan PDARs are designed for the detection and quantification of specific genetic sequences with assays, utilizing the 5' nuclease assay to quantitate target and control sequences in cDNA samples, and are conveniently supplied as primer and TaqMan probes mixes optimized for use. In this assay, we used β-actin as endogenous control. The relative quantitation of CD25 mRNA is assessed using the comparative C_T method by evaluating the C_T values for the unknown samples using the equation $2^{-\Delta\Delta C_T}$. We arbitrarily set the quantity of CD25 mRNA in lymphocytes of peripheral blood as one. All other samples are compared with peripheral blood.

Determination of soluble IL-2Rα by ELISA assay

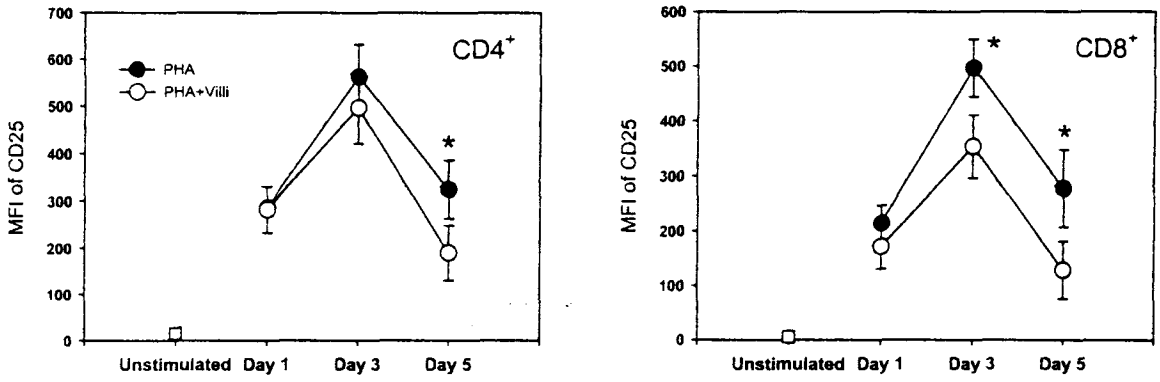
The shed sIL-2Rα in the supernatants were measured by ELISA assay. They were detected using commercial (R&D Systems, Minneapolis, MN USA; lower limit of sensitivity, <10 pg/mL) matched pairs of anti-human sIL-2Rα monoclonal antibodies (capture antibody and horseradish peroxidase-linked detecting antibody) optimized for use according to the manufacturer's guidelines. Briefly, the samples or standards were applied to a 96-well polystyrene microplate coated with a specific monoclonal antibody. At the same time, enzyme-conjugated detecting antibody was added. After a 3-hour incubation, the plate was washed to remove any unbound substances and stabilized hydrogen peroxide and chromogen (tetramethylbenzidine) were used to develop the colored reaction product, which was recorded as the optical density at a wave-length appropriate for the specific product using an ELISA plate reader. The sIL-2Rα concentrations were calculated from a standard curve generated with specific standards provided with each kit.

RESULT

Decreased IL-2Rα Expression on Activated T Cells when Cocultured with Villous Cells

During the coculture assay, PHA-activated PBMCs were cultured with autologous villous cells. The expression of CD25 and HLA-DR on activated T cells was measured by flow cytometry. The kinetic expression of IL-2Rα on activated T cells was compatible with the known physiological pattern, being highest on day 3 and having progressively decreased by day 5. However, the villous cells affected the expression of CD25 and HLA-DR by T cells in coculture system. On day 1 and 3, the MFI of IL-2Rα expression on activated $CD4^+$ cells was similar in both coculture and non-coculture groups. Nevertheless, the MFI of IL-2Rα on $CD4^+$ cells was significantly lower in coculture group than that in non-coculture group on day 5. It was noteworthy that this decrease on $CD8^+$ cells was noted since day 3.

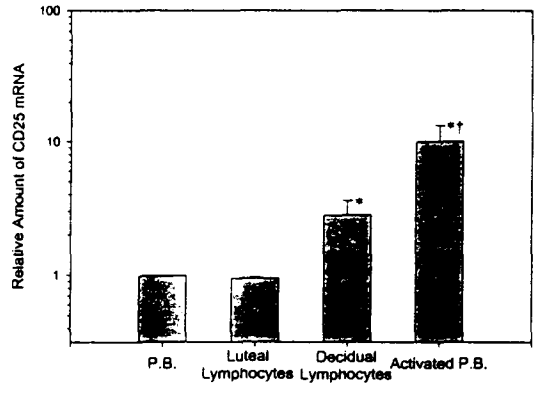
Fig 1 MFIs of CD25 expression on activated T cells when cocultured with villous cells (O, experimental group) or not (●, control group).



No Decrease in the Expression of IL-2Rα on Decidual T lymphocytes at the Transcriptional Level

Next, we studied whether the decreased expression of IL-2Rα on decidual T lymphocytes was mediated at the transcriptional level. The T cells were isolated through MACS with anti-CD19 antibody with a purity of 97% from decidual and endometrial lymphocytes, unstimulated PBMCs, and PHA-activated PBMCs.

Fig 2 The relative IL-2Rα mRNA levels in the T lymphocytes of peripheral blood (P.B.), luteal endometrium, deciduas, and PHA-actvated peripheral blood. The IL-2Rα mRNA level in resting T cells of peripheral blood was arbitrarily set to one. (* $p<0.05$ when compared with P.B.; † $p<0.05$ when compared with activated P.B.)



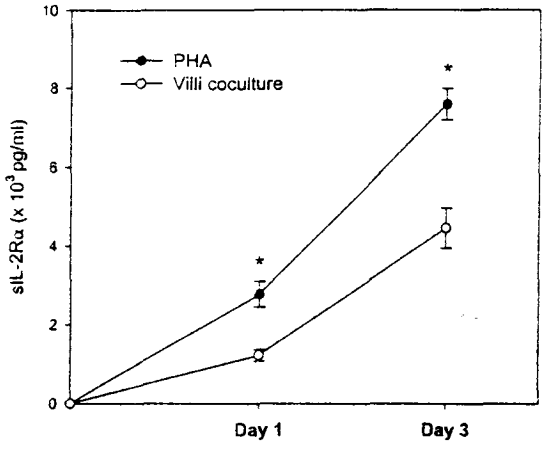
Expression of IL-2Rα mRNA was measured by real-time PCR. Although the amount of IL-2Rα mRNA in decidual T cells was not as much as that in fully activated T cells, it was about three-fold of that expressed in unstimulated PBMC-derived T cells and endometrial T cells during luteal phase. Because the expression of IL-2Rα mRNA was between that of unstimulated and fully stimulated T lymphocytes, the down-regulation of CD25 did not occur at the transcriptional levels.

Decreased sIL-2Rα release from Activated T Cells by Villous Cells

The IL-2Rα is enzymatically cleaved shed from the surface of expressing cells. It can be measured in the serum by ELISA assay as a 45 kDa soluble IL-2 receptor (sIL-2Rα). The release of sIL-2Rα is proportional to its cell surface expression. It is excreted and catabolized by the kidneys and has a serum half life ($t_{½}$) of 0.62 hours. In the Figure 3, we analyzed the amounts of sIL-2Rα in the supernatants of PHA-activated PBMCs and cocultured with villous cells. The mean concentrations of sIL-2Rα were increased about four-fold on day 3 when compared to that on day 1 in both groups. However, the mean concentration of sIL-2Rα in the supernatants of coculture group was significantly lower than that in PHA-activated group on both day 1 and 3. It meant that villous cells decreased the expression of IL-2Rα on the cell surface of

activated T cells. Whether this was due to over release of IL-2Rα from activated T cells, we need further more assays with protease inhibitors to clarify this possibility.

Fig 3 The levels of sIL-2Rα in the supernatants of PHA-activated PBMCs (●, control group) and cocultured with villous cells (○, experimental group).



DISCUSSION

Since half of the fetal genome derives from the father, the fetus may synthesize antigens considered to be foreign by the maternal immune system. Furthermore, the fetal cells, with immunogenic antigenic molecules, are frequently detected in the maternal circulating blood. It is presumed that these cells are released into the maternal blood during proliferation of trophoblast cells, following tissue ruptures that occur at the terminal extremity of the growing chorial villi, i.e. the fetomaternal interface. Although the maternal immune system comes into contact with these potential fetal immunogens at both systemic and local areas, the conceptus is tolerated and pregnancy can reach its term without any rejective problem in the greatest majority of cases.

As we know, immunologic rejection has been shown to require intact $CD4^+$ helper T cells. The stimulation of T-cell receptors with antigen or a mitogenic lectin induces a signal transduction pathway involving protein kinase C, which stimulates IL-2 secretion and upregulation of the α subunit of the IL-2 receptor (CD25). Secreted IL-2 functions in an autocrine and paracrine manner by binding to the full IL-2 receptor to initiate T-cell proliferation and differentiation. It has been shown that Induction of IL-2 gene transcription and expression of IL-2R precede acute rejection. To understand how immunity may be suppressed in the uterus during pregnancy, several experiments were performed to determine at what steps T-cell tolerance acts to inhibit lymphocyte proliferation. We tested the effects on mitogen-stimulated CD25 expression, mitogen-stimulated IL-2Rα gene expression, and the release pattern of sIL-2Rα. In the future, we will further test the effects of protease and protease inhibitors, as well as the responsiveness to human IL-2.

In previous study, decreased expression of CD25 was found on activated $CD4^+$ and $CD8^+$ T lymphocytes derived from decidual tissue, although these lymphocytes expressed high levels of CD69 and HLA-DR antigens. Now we demonstrate that the expression of IL-2Rα on the surface of activated T cells is downregulated when cocultured with villous cells. Together, these finding indicate that villous cells can reduce IL-2Rα expression on encountered T cells in a specific manner. However, the decreased CD25 expression on decidual lymphocytes dose not affect steady-state amounts of IL-2Rα mRNA, which even approaches that in fully-activated T cells. The similar condition is present in tumor-infiltrating lymphocytes from the tissue of cervical cancer and breast cancer. The failure of downregulation of IL-2Rα mRNA in decidual lymphocytes and tumor-infiltrating lymphocytes would suggest that immune suppression during pregnancy and tumor formation did not occur at the transcription level and it might be due to excessive shedding of IL-2Rα from T cells.

Serum concentration of the soluble form of the IL-2Rα has been found to be increased in patients rejecting allograft. The increase of sIL-2Rα in the supernatants reflects the increase of IL-2R on the surface of T lymphocytes, which in turn affects the T-cell proliferation mediated by IL-2. Nevertheless, whether the villous cells could enhance the degradation of IL-2Rα on activated T cells and whether a proteolytic cleavage might mediate the process, we need further studies to clarify the exact mechanism.