行政院國家科學委員會專題研究計畫　成果報告

蛻膜淋巴球細胞動力素 IL-15 和 IL-15 接受器的表現

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摘要

我們先前的研究發現，著床後蛻膜T細胞的活化標誌（CD69、HLA-DR、CD38 和CD71）會增加，但是CD25的表現卻選擇性地減少。因為CD25是與IL-2接受器有關的表面活化抗原，其與淋巴球的活化與增殖有關，CD25表現的減少可能意謂「細胞毒殺淋巴球株」不易增殖。此發現正可以解釋懷孕婦女對胎兒抗原有降低免疫反應發生的現象，這些都與母體-胎兒界面沒有排斥發生以及妊娠成功有關。然而，過去的研究在母體-胎兒界面一直沒有發現IL-2的存在。

1994年新發現之細胞動力素IL-15，其能刺激IL-2接受器，使淋巴球活化與增殖，讓我們對蛻膜T淋巴球上CD25之減少又有了新的認識。在此研究中，我們發現蛻膜淋巴球上也有IL-15接受器α的存在，其密度與周邊血液中的淋巴球差不多。雖然子宮內膜間質細胞與巨噬細胞分泌豐富的IL-15，但是蛻膜T淋巴球上IL-2Rα和IL-15Rα的表現受到抑制，於是蛻膜淋巴球就不易活化，排斥反應將不會發生，這正可以說明正常懷孕時母體並不會排斥胎兒。

此外，我們也證實若發生萎縮卵，蛻膜T淋巴球上的IL-15Rα與它的mRNA將會增加，於是細胞毒殺淋巴球和NK細胞產生，流產於焉發生。這些在在說明調控IL-15和其接受器，對維持正常懷孕，扮演一重要角色。

Keywords

IL-15, IL-15Rα, decidual T cells, anembryonic pregnancy

Introduction

A successful pregnancy requires the maternal immune system to accept the immunologically semiallograft fetus. Although the key factors and precise mechanisms involved in this process are not clearly understood, it has been proposed that cytokines are one of the mediator of maternal immunity reactivity [1-4]. Cytokines and growth factors are known to play a pivotal role, not only in modulating maternal immune response, but also in regulating other response, e.g. implantation [5], trophoblast invasiveness and tissue remodeling during placental development [6], and finally in labor [7]. Interleukin-2 (IL-2) is known for its strong proliferating and activating function on lymphocytes and the expression of IL-2 receptor (IL-2R) makes a critical step in the activation of T cells. Therefore, it is involved in allograft rejection. In our previous study, we demonstrated that selective down-regulation of CD25 (IL-2Rα) on decidual T lymphocytes [8]. The reduction in $CD25^+$ T cells during pregnancy might count for no rejection at the fetomaternal interface. However, it is difficult to detect IL-2 in nonpathologic human endometrium and decidua [9-11]. It appears that IL-2 is not produced anywhere in the normal implantation site.

Recently, the cytokine IL-15 was discovered as T-cell growth factor activity secreted from a monkey kidney epithelial cell line [12]. Its molecular structure and biological function are similar to those of IL-2, but its sequence and tissue localization are quite different from those of IL-2. IL-15 can proliferate the entire lymphocyte population, including B cells, T cells and NK cells [13-15].

IL-15 receptor (IL-15R) also resembles IL-2R because it is composed of three subunits: IL-15Rα, the molecule binding site; IL-2Rβ and the common γ chain, which is the signal transduction site [16, 17]. These similarities between IL-2 and IL-15 suggest that IL-15 may be a potent effector of uterine T and NK cells. In this study, the expression of IL-15, IL-15Rα and their mRNA in human peripheral blood and first trimester decidua were investigated.

Materials and Methods

Subjects

Ten healthy pregnant women who had elective abortions of normal pregnancies due to multiparity and ten women who had anembryonic pregnancy (blighted ovum) between 6 and 10 weeks of gestational age were enrolled in the study with informed consent and under the approval of the investigation review board of our hospital. Anembryonic pregnancy was identified by sonography that failed to show a fetal pole when the gestational sac was greater than 25 mm. None of the pregnant subjects was receiving any medication, and all were nonsmokers. Subjects with associated complications, which were judged by clinical examination and general laboratory tests on pregnant women, and those with subclinical autoimmune diseases, which were judged by serological tests, including anti-nuclear antibody, rheumatoid factor, and anti-thyroid-microsomal antibody, were excluded from this study. The two groups were matched by the number of previous pregnancies and body mass index.

Specimens

Decidual tissue 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: 1g/L D-glucose, 0.35g/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 (Amersham Biosciences, Uppsala, Sweden)

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 were also isolated by Ficoll-Paque PLUS sedimentation.

Immunophenotypic and Intracellular Cytokine Analysis

Peripheral blood and decidual mononuclear cells were stimulated with Phorbol 12-Myristate 13-Acetate (PMA) plus ionomycin in the presence of Brefeldin A (BFA) at 37°C and 7% CO_2 for 4 hours. The final concentrations of the stimulants used in the culture were: PMA 0.1mg/mL and Ionomycin 0.5mg/mL. BFA was used at a final concentration of 5mg/mL to block intracellular transport within the Golgi complex and retain the cytokines within the cells.

The anti-human IL-15 monoclonal antibody (mAb) was used. The following cell-surface antigen-staining mAbs, labeled with fluorescein-5-isothiocyanate (FITC), phycoerythrin (PE), or peridinin chlorophyll (PerCP), were obtained from Becton Dickinson Immunocytometry Systems (Becton Dickinson; San Jose, CA USA): anti-CD3, anti-CD4, anti-CD8 and anti-IL-15Rα. About 1×10^6 mononuclear cells were stained at 4°C in the dark for 20 min with different mAbs to determine surface phenotype. FACS Lysing Solution was added to lyse the red cells. Afterward the cells were washed twice and resuspended with IL-15-specific antibodies and FACS Permeabilizing Solution to stain intracytolplasmic cytokines.

List mode data were acquired on a FACScan cytofluorimeter (Becton Dickinson) and analyzed using CellQuest software. Dead cells and monocytes were excluded by forward (FSC) and side (SSC) angle scattered light gating. Typically, 5,000 events were acquired in the gating window. In this tight window, we assessed that most of the cultured cells were $CD3^+$. Two parameter dot plots showing cytokine staining were then created; quadrants were placed according to the staining of the negative control, i.e., cells stained with IgG1+IgG2a mAbs. All percentages listed in the text and the tables represent positive net percentages.

Quantitation of IL-15 and IL-15Rα 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-15 and IL-15Rα mRNA is assessed with TaqMan PCR method. The method is based on the use of fluoregenic probes that anneal 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 reporter 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 samples 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 IL-15 and IL-15Rα cDNA is linear under optimum conditions. The more the initially starting copy number of cDNA, the less the C_T value.

In this assay, we used β-actin as endogenous control. The relative quantitation of IL-15 and IL-15Rα 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 IL-15 and IL-15Rα mRNA in lymphocytes of peripheral blood as one. All other samples are compared with peripheral blood.

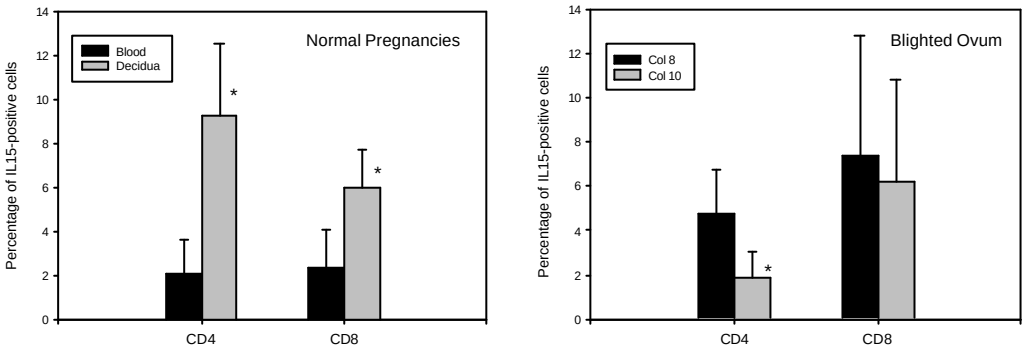
Statistical Analysis of Data

All results were expressed as mean ± SE unless stated otherwise. Data analysis was performed with SPSS for Windows software (SPSS Base System, SPSS Inc., Chicago, IL) using independent and pared *t*-test. A value of $P<0.05$ was considered statistically significant.

Results

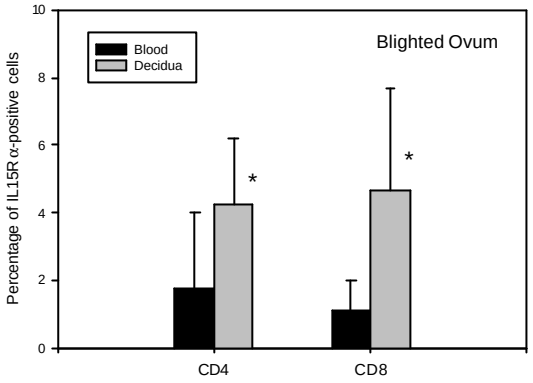
Increased Percentage of IL-15-Positive Cells in Decidual T Lymphocytes of Normal Pregnancy, but not in Anembryonic Pregnancy

In normal pregnancy, the percentage of IL-15-positive cells was increased significantly in decidual T lymphocytes than peripheral blood, irrespective of $CD4^+$ or $CD8^+$ cells (Fig. 1). However, this phenomenon disappeared in anembryonic pregnancy. Even the proportion of IL-15-positive cells was decreased markedly in decidual $CD4^+$ lymphocytes.



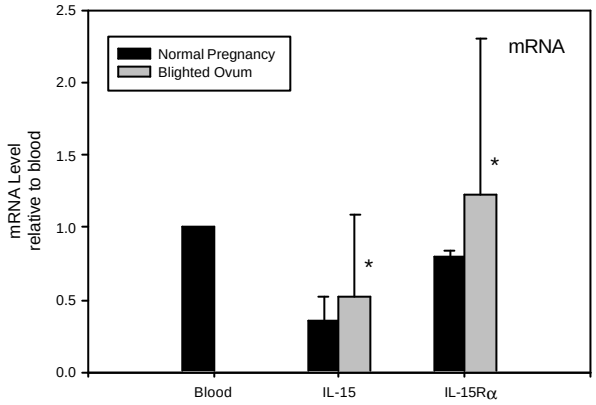
Increased Percentage of IL-15Rα-Positive Cells in Decidual T Lymphocytes of Anembryonic Pregnancy, but not in Normal Pregnancy

In anembryonic pregnancy, the proportion of IL-15Rα-positive cells in decidual T lymphocytes was increased markedly over that in peripheral blood (Fig 2). Comparison of IL-15Rα-Positive Cells in decidual T lymphocytes of normal pregnancy with that in peripheral blood showed no significant differences. (data not shown)



Increased Levels of IL-15 and IL-15Rα mRNA in Anembryonic Pregnancy than Normal Pregnancy

The technique of real-time PCR of human cDNA expression was used to identify differences in the expression pattern of genes in decidua between normal pregnancy and anembryonic pregnancy. Fig 3 showed the most prominent changes observed was an increase of both IL-15 and IL-15Rα mRNA in anembryonic pregnancy.



Discussion

Historically, IL-2 was known as a "T-cell growth factor". IL-2 was originally isolated from T-cell culture supernatants and was shown to expand and support the growth of T cells in vitro [18]. The role of IL-2 as a growth factor stemmed initially from *in vitro* work, due to its activating and expanding potential for T cells [19]. However, there was no IL-2 detected at the fetomaternal interface. Furthermore, we demonstrated that the expression of IL-2 receptor on the decidual T lymphocytes was selectively down-regulated during normal pregnancy. These might explain why the mother did not reject her semiallograft fetus.

Recently the IL-15 was cloned and sequenced from simian kidney epithelial cells CV-1/EBNA [12] and from human adult T cell leukemia cell line HuT-102 [20]. It is a member of the four α helix bundle cytokine family, which binds to and induces signaling through the IL-2 receptor β and γ chains [21]. IL-15 shares a number of biological activities with IL-2. IL-15 acts as a T cell stimulant and plays a pivotal role in cell-mediated immunity by activating T cell proliferation and B cell antibody production, and by promoting natural killer cell cytotoxicity [13, 14]. However, the expression of mRNA for IL-15 has been seen in many other cell types [12]. The broad expression of

mRNA encoding IL-15 compared with the expression of IL-2 suggests that IL-15 has activities beyond the immune system. For example, IL-15 enhances the invasion and migration of cytotrophoblastic cells (JEG-3) *in vitro* [22]. These findings suggest the importance of IL-15 at the fetomaternal interface during early pregnancy.

In this study, it was demonstrated that the expression of IL-15 was increased in the decidual T lymphocytes during normal pregnancy. IL-15 is involved in regulating the differentiation of granulated metrial gland (GMG) cells during mouse pregnancy [23, 24]. GMG cells belong to the NK cell lineage and have been identified in human as uterine NK cells [25, 26]. Uterine NK cells are present in large numbers in human decidua and thought to play an important role in the maintenance of pregnancy. Therefore, increased proportion of IL-15-positive cells in human early pregnancy implies an important role of this cytokine in the regulation of $CD16^{-}CD56^{bright}$ NK cells in human endometrium. Furthermore, we demonstrated that the expression of IL-15Rα in decidual T lymphocytes was not increased markedly. The suppressed levels of both IL-2Rα and IL-15Rα meant that the cytotoxic T and NK cells would not be activated although the amount of IL-15 was increased. As a result, the maternal immune system would not be hostile to the fetus.

However, when anembryonic pregnancy occurred, in one hand, the production of IL-15 was decreased and, in the other hand, the expression of IL-15Rα and its mRNA was increased. These might facilitate the development of cytotoxic T and NK cells. Therefore, rejective process exploded at the fetomaternal interface and abortion was followed. This study indicated that IL-15 was an important cytokine in maintenance of the pregnancy through adjusting the levels of IL-15 and its receptor.

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