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Investigation of plasma cytokine levels and endometrial tissue leukocytes in recurrent pregnancy loss

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Abstract

Immigrant and resident leukocytes are the main immune components of endometrial and decidual tissue. Altered immune responses have been suspected to be involved in idiopathic recurrent pregnancy loss (RPL) pathogenesis. We aimed to investigate the plasma cytokine levels and endometrial leukocyte subpopulations of RPL cases. This study was conducted with 60 idiopathic RPL (28 primary and 32 secondary) cases. Peripheral venous blood samples were drawn and hysteroscopic investigation of uterine cavity with endometrial samplings were performed during mid-follicular phase. IL-17, IFN-gamma, TNF-alpha, TGF-beta, IL-6, IL-4, IL-10, SOCS3 and IL-35 plasma levels were assayed by enzyme linked immunosorbent assay. CD4, CD8, CD56 and CD163 staining of endometrial tissue was performed with immunohistochemistry method. The comparison of plasma IFN-ɣ, IL-35, IL-17, SOCS-3, TGF-β, IL-6, TNF-α, IL-10 and IL-4 levels between primary and secondary RPL cases revealed no significant difference. The major staining of endometrial tissue was composed of CD8 antigen. The second dominancy was observed with CD56 antigen. CD163 antigen staining was lower than CD8 and CD56 staining. CD4 positive cells were occasionally found. CD8 and CD56 positivity of secondary RPL cases were significantly higher than those of primary RPL cases. There were interaction between IL-10 levels and CD163 staining and TNF-α levels and CD56 staining. In conclusion; natural killer cells, macrophages and T supressor cells came into prominence on endometrial samplings of our RPL cases. And it is not possible yet to make definite consequences on RPL immunology with our results.

Keywords: Recurrent pregnancy loss, Natural killer, Macrophage, Endometrial leukocytes, Cytokines

1. Introduction

Maternal immune system is closely controlled to tolerate an embryo and a fetus with semiallogenic paternal antigens during pregnancy. Furthermore, immune-mediated processes such as tissue growth, remodeling, and differentiation are essential to maintain pregnancy (1). Limited HLA expression on trophoblasts (2), changes of lymphocyte profiles and activity (3), the balance and shift of T helper1 (Th1) (cell-mediated immunity) and Th2 (humoral immunity) cytokines, and an increase of regulatory T cells (4) have been proposed as possible mechanisms for immune tolerance. Dysregulation of this exhaustive immune control may lead reproductive failure, such as implantation failure, pregnancy loss, preterm birth, intrauterine fetal growth restriction, and preeclampsia.

Recurrent pregnancy loss (RPL)defines as three or more spontaneous pregnancy losses before 20 weeks of gestation (5). The etiology of RPL is multifactorial and many causes such as genetic, endocrine,

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anatomic, infectious, and immune factors have been proposed. Approximately half of the cases of RPL are classified as idiopathic (6, 7). Recently, altered immune responses have been suspected to be involved in idiopathic RPL pathogenesis (8, 9).

Immigrant and resident leukocytes are the main immune components of endometrial and decidual tissue (10, 11). Three principal populations of leukocytes are; macrophages with CD163 antigen, large granulated lymphocytes of the natural killer (NK) cell lineage, most of which bear CD56 antigen, and T lymphocytes positive for CD4 and CD8 antigen (10, 12-15).

Development, differentiation, and maintenance of these cells are orchestrated by 2 key signaling axes: the antigen-specific cell receptor and cytokine-mediated signals (16). It was reported that endometrial NK cells are differently regulated in women with idiopathic RPL (17). The lost of NK cell-mediated immune regulation and coupling with an elevated T helper17 response and extensive local inflammation are the components of immune alteration in RPL (18).

In this study we investigated the plasma levels of cytokines and endometrial leukocyte subpopulation staining pattern in idiopathic RPL cases.

2. Materials and Methods

2.1. Study design

This study was conducted at IVF Unit of our university hospital during October 2011 and May 2013 after approval of local ethical committee. A total of 60 voluntary women with a history of two or more pregnancy losses, at least two consecutive, before 20 weeks of gestation, between the ages of 19-39 years, were enrolled in the study. Among these 60 women, 28 women without history of alive delivery were accepted as primary RPL and the remaining 32 women with history of alive delivery were accepted as secondary RPL.

The etiology of all RPL cases were idiopathic and this was determined by exclusion of genetic, anatomic, endocrine and autoimmune factors. Clinical evaluation for RPL includes the following: karyotyping from couples, hereditary and acquired thrombophilia evaluation (FV Leiden, FII, MTHFR and PAI-1 polymorhisms; protein C, protein S and antitrombin-III levels), pelvic ultrasonography, hysteroscopy, thyroid function test, serum hemoglobin a1c level and antiphospholipid antibodies (lupus anticoagulant, IgG and IgM anticardiolipin).

Table 1. Details of immunohistochemical analysis

Primary Antibody	Manufacturer	Clone	Dilution	Pretreatment
CD4	Leica-Novocastra (Germany)	1F6	1:20	Microwave pressure cooker, 10 min, EDTA
CD8	Thermo (USA)	SP16	1:50	Microwave pressure cooker, 10 min, 10mM citrate buffer (pH 6.0)
CD56	Thermo (USA)	123C3.D5	1:100	Microwave pressure cooker, 20 min, 10mM citrate buffer (pH 6.0)
CD163	Leica-Novocastra (Germany)	10D6	1:100	Microwave pressure cooker, 10 min, 10mM citrate buffer (pH 6.0)

2.2. ELISA assay

Peripheral venous blood samples were drawn during the mid-follicular phases of a menstrual cycle and the samples were centrifuged at 2500 rpm at 4°C for 15 minutes, and stored at -20°C until analysis. The extracted plasma samples were assayed by an enzyme linked immunosorbent assay (ELISA) using commercially available kits for IL-17, IFN-gamma, TNF-alpha, TGF-beta, IL-6, IL-4, IL-10 (Boster Biological Fremont/CA) and SOCS3 and IL-35 (USCN Wuhan/China) according to the manufacturer's instructions. The samples were analysed by the same staff in the same laboratory conditions. Within and between assay variations were less than 6% and 8% for all ELISA assays, respectively.

2.3. Endometrial Sampling and Immunohistochemical Study

Hysteroscopic investigation of uterine cavity and endometrial sampling was performed during mid-follicular phase of menstruation on all RPL cases. Tissues were fixed in the neutral 10% formaldehyde, embedded paraffin, cut in 5-μm sections and stained with Hematoxilen-Eosin. For immunohistochemical staining, 5 μm paraffin sections were deparaffinized in xylene, rehydrated and then placed in a phosphate buffer saline (PBS) bath (pH 7.6). Antigen retrieval was performed using a 15-min bath in boiling citrate buffer (Ph 6.0) solution. Sections were treated with 3% hydrogen peroxide for 5-min to quench endogenous peroxidase activity, rinsed with

deionized water and then placed in the PBS. Sections were incubated first with 1% pre-immune rabbit serum to reduce non-specific staining and then monoclonal antibodies to CD4, CD8, CD56 and CD163 for 45-60 minute each at room temperature (Table 1). Immune detection was performed using a biotin-streptavidin detection system (BioGenex, San Ramon, CA) with 3,3'-diaminobenzidine chromogen (Dako, Carpinteria, CA). Tissues were counter-stained with Mayer's hematoxylin, dehydrated and then cover-slipped with permount on glass slides and then evaluated under a light microscope. Positive cells were counted randomly at 320 x magnification in 3 fields.

2.4. Statistical analysis

Statistical analysis were performed by Statistical Package for Social Sciences 16.0 (SPSS Inc., Chicago, IL, USA) version. Results were presented as mean ± SEM. Differences in continuous variables between groups were analysed by Student's *t*-test or Mann Whitney U test according to distribution of data. The differences for categorical variables were analysed using the chi-square test or Fisher's exact test, where applicable. The relations among plasma cytokine levels and endometrial leukocyte antigens were evaluated with Pearson correlation and regression analysis. P values of <0.05 were considered as statistically significant.

3. Results

The comparison of demographic characteristics between primary and secondary RPL cases revealed no significant difference (Table 2).

The comparison of serum IFN-ɣ, IL-35, IL-17, SOCS-3, TGF-β, IL-6, TNF-α, IL-10 and IL-4 levels between primary and secondary RPL cases were presented in Table 3. There was no significant difference between primary and secondary RPL cases in these parameters.

Immunohistochemical staining results of CD4, CD8, CD56 and CD163 antigens on endometrial tissues were presented in Table-4. The major staining of endometrial tissue was composed of CD8 antigen. The second dominancy was observed with CD56 antigen. CD163 antigen staining was lower than CD8 and CD56 staining. CD4 positive cells were occasionally found and the comparison of the staining of these antigens between primary and secondary RPL cases indicated significant difference for CD8 and CD56 staining. CD8 (p=0.02) and CD56 (p=0.04) positive cell numbers of secondary RPL cases were significantly higher than those of primary RPL cases.

The correlation analysis of CD8, CD56 and CD163 between each other revealed no relation. The correlation analysis between plasma cytokine levels and endometrial tissue leukocyte antigen staining revealed positive relations between; 1-) CD163 staining and SOCS3 plasma level (R=0.27, p=0.04) and CD163 staining and IL-10 plasma level (R= 0.43, p<0.01), 2-) CD56 staining and TNF-α plasma level (R= 0.34, p=0.01). The linear regression analysis showed the influence of plasma IL-10 level on endometrial CD163 staining as OR=10, p<0.01, CI= 1.71- 2.48; and the influence of plasma TNF-α level on endometrial CD56 staining as OR=9.5, p<0.01, CI= 0.37- 0.57.

Table 2. Comparison of demographic characteristics of primary and secondary RPL cases

Parameter	Primary RPL (n=28)	Secondary RPL (n=32)	P value
Age (years)	30.1±4.7	33±5.2	0.03
Gravid	2.7±0.9	4.2±2.2	<0.01
Abortion	2.7±0.9	2.8±1.8	0.69
BMI (kg/m2)	24.9±5.2	27.1±4	0.1
D3 FSH (IU/L)	7.2±3.2	8.1±3.7	0.3
D3 Estradiol (pg/mL)	43.1±18.5	50.9±37.4	0.4
TSH (IU/L)	1.4±0.9	1.9±1.6	0.1
Prolactin (µg/L)	16.8±12.2	9.5±3	<0.01
Smoker (%)	21.4	17.2	0.6
History of allergy (%)	7.1	3.4	0.5
Menstruation pattern (%)			
-regular	70.4	89.7	
-meno/metrorrhagia	7.4	3.4	0.07
-oligomenorrhea	22.2	6.9	

Values are presented as mean±SD. BMI= body mass index; D3= the third day of menstruation

Table 3. Comparison of plasma cytokine levels of primary and secondary RPL cases

Cytokine	Primary RPL (n=28)	Secondary RPL (n=32)	*P* value
IFN-γ (ng/mL)	27±3.23	32.69±7.54	0.3
IL-35 (ng/mL)	52.56±3.85	56.32±4.8	0.5
IL-17 (ng/mL)	57.52±11.37	67.84±10.86	0.5
SOCS3 (ng/mL)	5.21±0.63	9.75±4.02	0.2
TGF-β (ng/mL)	36.69±7.2	57.03±17.25	0.2
IL-6 (ng/mL)	6.17±1.33	7.21±1.45	0.6
TNF-α (ng/mL)	28.96±5.06	39.11±5.65	0.1
IL-10 (ng/mL)	66.33±8.72	66.52±7.36	0.9
IL-4 (ng/mL)	18.47±1.68	18.03±1.73	0.8

Values are presented as mean±SEM

Table 4. Immunohistochemical study of endometrium in primary and secondary RPL cases

Antigen	Primary RPL[a](n=28)	Secondary RPL[a] (n=32)	P value
CD 163	25.89±18.96	26.71±16.38	0.86
CD 56	59.78±25.95	75.65±30.96	**0.04**
CD 8	76.31±19.66	94.52±34.28	**0.02**
CD 4	±	±	

Note: [a] = Mean number and standard deviation of positive cells in 3. random fields (320 x). ±= Occasionally found positive cells.

4. Discussion

In this study we observed high CD8(+) cytotoxic T lymphocyte and CD56 (+) NK cell staining on follicular phase endometrial tissues of RPL cases. The staining of these leukocytes were significantly high on endometrial tissue of secondary RPL cases. Also we observed influence of plasma IL-10 levels on endometrial CD163(+) macrophage staining, and influence of plasma TNF-α levels on endometrial CD56(+) NK cell staining. Limitation of our study was the absence of follicular phase endometrial samplings of fertile women. During approval of ethical committee, permission for tacking endometrial sampling from age-matched fertile women was not given. We did not use the endometrial samplings of women undergoing gynecological surgery for benign indications to prevent the bias.

Survival of the allogeneic embryo in the uterus depends on the continuation of immune tolerance at the maternal-fetal interface (18). Cellular immune responses particularly directed by NK and T cells are often dysregulated in RPL. Excessive or inappropriate accumulation of NK cells in uterus may lead to cytotoxic environment and may lead to disruption of trophoblastic proliferation and differentiation. (19). Uterine NK cells express CD56 antigen at a higher level than peripheral blood NK cells. Papamitsou et al. observed high CD56 antigen expression on decidual tissue of RPL cases compared to decidual tissue of age-matched women electively terminated their pregnancies during the first trimester of pregnancy (20). Giuliani et al. studied CD56 expression on mid-secretory phase endometrial tissue of 21 endometriotic RPL cases and 10 fertile controls with immunohistochemistry method. Comparison of the average percentage of CD56 (+) cells between endometriotic RPL cases (18.3 ±14.6) and fertile controls (22.3±19.9) revealed no significant difference (21). Fu et al. found that decidual NK cells promote immune tolerance during pregnancy by supressing inflammatory T helper 17 cells via IFN-γ secretion. Defective NK-cell–mediated regulatory response in RPL patients results in a different T helper 17 response and extensive local inflammation (22). In our previous study we observed significantly high plasma IL-17 levels and significantly low plasma IL-35 levels in RPL cases compared to those of fertile women. IL35/IL17 ratio was significantly low in RPL group compared to that in fertile controls (23). In our current study we detected no difference for plasma IL-17 and IL-35 levels between primary and secondary RPL cases, but endometrial CD56 (+) staining was significantly higher in secondary RPL cases than primary RPL cases. In our opinion this difference may be important in the explanation of alive delivery in the secondary RPL group. Interestingly Quenby et al. suggested that the relation between reproductive failure and increased uterine NK cell density might be explained by excessive oxidative stress due to increased angiogenesis during

implantation period (24, 25).

Yoo et al. suggested that reproductive failures in women with RPL may become from immune disregulation between CD8(+) T cells and NK cells (26). Darmochwal-Kolarz et al. compared the expression pattern of peripheral blood T suppressor CD8(+) lymphocytes between 14 idiopathic RPL and 18 fertile women with flow cytometric method. T suppressor CD8(+) lymphocytes were lower in women with pregnancy failures in comparison with fertile women (27). Malinowski et al. analyzed peripheral blood lymphocyte subpopulations in 117 idiopathic nonpregnant RPL women in comparison with 44 healty multigravid nonpregnant women. The percentage of CD8(+) lymphocytes of RPL group was lower (20.2% vs. 23.9%) and the CD4+/CD8+ ratio of RPL group was higher (2.37 vs. 1.9) than those of fertile healty women. They also observed no significant difference in the distribution of lymphocyte subpopulations between primary and secondary RPL cases (28). Lachapelle et al. observed similar findings. The percentage of endometrial CD8(+) T lymphocytes was significantly decreased in recurrent aborters, and their CD4:CD8 ratio was increased (29). In our study, CD8(+) endometrial leukocyte counts of secondary RPL cases were significantly higher than the count of primary RPL cases. Vassiliadou and Bulmer investigated CD4 and CD8 staining on decidual biopsy specimens of 18 elective termination and 20 spontaneous abortion by immunohistochemistry method. They reported that endometrial CD4(+) and CD8(+) cell staining numbers and proportions which have been demonstrated in normal early pregnancy were similar to spontaneous abortion, but the staining dominancy was observed on CD8 positivity (30). But, in our study, CD4(+) cell staining was rare. Interestingly Lea et al. reported that 50% of 14 recurrent miscarriage patients showed a lack of suppressor cells on decidual biopsy specimens and 59% were subnormal in comparison to 20 normal pregnant controls (31). Bao et al. studied CD4(+) T cells on decidual tissues of 21 idiopathic RPL cases and 30 elective termination cases during first trimester. Decidual lymphocytes were collected by Ficoll density gradient centrifugation. The frequency of CD4(+) cells was decreased in idiopathic RPL decidua compared to controls. They suggested that suppression function of T cells was mediated predominantly through IL-10 and TGF-β in decidua (32). In our study, we observed the influence of plasma IL-10 levels on endometrial CD163(+) macrophage staining, and influence of plasma TNF-α levels on endometrial CD56(+) NK cell staining. Kwan et al. investigated the dynamic change of maternal decidual leukocyte populations on elective termination cases from first to second trimester with multicolor flow cytometric method. They observed increament of CD163(+) macrophages from first to second trimester (33). Abumaree et al. observed the increament of IL-10 secretion during CD163(+) macrophage differentiation (34). Russell et al. examined the endometrial leukocytes of 222 RPL and in vitro fertilization failure cases. CD8(+) T-cells showed focal perivascular aggregates in most instances. CD163(+) cells were distributed evenly throughout the superficial endometrial stroma and also present as single or clustered macrophages within the lumens of superficial glands, mostly in the luteal phase. CD56(+) NK cells showed diffuse but variable distribution throughout the functional layer and perivascular aggregates of various sizes in two thirds of cases. They demonstrated the co-location of CD8(+) T-cells and CD56(+)NK cells in perivascular aggregates during luteal phase (35). Quenby et al. reported that RPL cases had significantly more CD4(+), CD8(+),and CD56(+) leukocytes in their mid-luteal phase endometrium than either those who had live births or women with proven fertility (36).

In conclusion; NK cells, macrophages and T supressor cells came into prominence on endometrial samplings of our RPL cases. But it is yet not possible to make a desicion for determining the responsible immunologic factor to explain RPL. To discuss our results expanded population studies with fertile controls are needed.

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