Deerased ratios of serum Th17/Treg-related cytokines in women with a defect in implantation after in vitro fertilization

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Objective: To compare the ratios of serum T-helper type 17 (Th17) cell/regulatory T (Treg) cell related cytokines between non-pregnant women with repeated implantation failures (RIFs) after in vitro fertilization and embryo transfer (IVF-ET) cycles due to a defect in implantation and those with normal fertility (controls).

Methods: Enzyme-linked immunosorbent assay (ELISA) was used to measure the concentrations of IL-17, IL-6, IL-23, TGF-β and IL-10 in the serum of 28 women with RIF and 23 normal fertile women.

Results: The ratios of Th17/Treg related cytokines, including the ratios of IL-17/IL-10, IL-23/IL-10, IL-23/TGF-β and IL-6/IL-10, were significantly lower in women with RIF due to a defect in implantation than those in controls.

Conclusion: Decreased ratios of serum Th17/Treg related cytokines may play a role in the pathogenesis of a defect in implantation failure.

Key words: T-helper type 17 cell, regulatory T cell (Treg), cytokine, implantation failure

Introduction

Successful human in vitro fertilization (IVF) and embryo transfer (ET) may still result in a low implantation rate. In addition, a certain number of idiopathic sterilities are due to repeated implantation failures. This failure may be due to a defect of implantation as no human chorionic gonadotrophin (hCG) production is ever detected, or an occult pregnancy loss. It may also occur after a transient detection of hCG production, following which an immediate drop of hormone levels can be detected, signifying to early pregnancy loss. During human pregnancy, a semi-allogeneic fetus implants in the uterus. At the feto-maternal interface, inflammatory processes can take place due to the invasion of micro-organisms [1], but also due to a sterile maternal immune reaction against allo-antigens on the fetus or trophoblast [2]. For some years a prevalent theory held that a predominant production of so-called T-helper type 2 (Th2) cytokines, such as interleukin (IL)-4 and IL-10, was a characteristic of normal pregnancy, whereas in miscarriage and recurrent miscarriage (RM) there was a predominant production of Th1 cytokines, such as interferon (IFN)-γ and IL-2 [3]. Th2 cytokines are known to direct the immune reaction towards a humoral response, which may not harm pregnancy, whereas Th1 cytokines direct the reaction towards a cytotoxic response. Elevation of different cytokine concentrations in the maternal serum proved that IFN-γ positive patients had twice the
risk for poor IVF pregnancy outcome compared with IFN-γ negative subjects, including a significantly lower implantation rate [4]. Another study found that both women with recurrent miscarriage (RM) and women with RIF after IVF had significantly higher ratios of tumor necrosis factor (TNF)-α/IL-4 and TNF-α/IL-10-producing T-helper lymphocytes in peripheral blood before pregnancy or IVF treatment compared with multiparous women [5].

However, recent studies have shown that the Th1/Th2 hypothesis is probably too simplistic [6,7]. Some degree of systemic inflammatory activation has been found to be a normal feature of at least the third trimester of pregnancy [8], whereas an increased degree of inflammation may characterize pre-eclampsia [8,9]. Thus, some degree of uterine or systemic inflammation appears to be necessary from implantation to birth. However, if inflammation during implantation becomes too weak or excessive, implantation failure may occur. Recently, CD4+CD25+ regulatory T (Treg) cells and Th17 cells have been described as two distinct subsets from Th1 and Th2 cells, respectively. Treg cells expressing the forkhead/winged helix transcription factor (Foxp3) have an anti-inflammatory role and maintain tolerance to self components by contact dependent suppression or by releasing anti-inflammatory cytokines (IL-10 and transforming growth factor [TGF]-β) [10], while Th17 cells expressing retinoic acid related orphan receptor γt (RORγt) play critical roles in the development of autoimmunity and allergic reactions by producing IL-17 and, to a lesser extent, TNF-α and IL-6 [11]. Thus, the balance between Th17 and Treg cells may be important in the development/prevention of inflammatory and autoimmune diseases [12].

Of the women with repeated implantation failures after IVFs in this study, one subgroup always had a negative hCG test, which suggests a defect in implantation. We postulate that inadequate inflammation may be one of the causes of this implantation defect. However, it is unclear whether there is a pre-existing imbalance of Th17-related cytokines (IL-17, IL-6, IL-23) and Treg-related cytokines (IL-10, TGF-β) in this subgroup. The purpose of this study was to compare serum Th17/Treg-related cytokines, including IL-17, IL-23, IL-6, IL-10 and TGF-β, and their ratios between non-pregnant women with repeated implantation failures (RIFs) after in vitro fertilization and embryo transfer (IVF-ET) cycles due to a defect in implantation (hCG negative) and those with normal fertility during late follicular phase.

Patients and methods

The study design was a prospective controlled study. This study protocol was approved by the Ethics Committee for Clinical Research at Taichung Veterans General Hospital (TCVGH), and informed consent was obtained from each participant. The study group comprised 28 women with a history of repeated implantation failures (RIF) with negative hCG after IVF-ET cycles who visited the gynecology outpatient department at TCVGH and were evaluated by a gynecologist (M-J Chen). They received thorough history taking and examinations including physical examinations, ultrasonography, hysterosalpingography (HSG) and blood tests of follicle-stimulating hormone (FSH), luteinizing hormone (LH), free thyroxine (free T4), thyroid stimulating hormone (TSH), prolactin, antinuclear antibodies, anticardiolipin antibodies, lupus anticoagulant, antithyroglobulin and antimicrosomal antibodies. Controls were volunteers. All controls were interviewed by a rheumatologist (H-H Chen), during which time personal and family histories were ascertained. Participants in the study group and the control group were enrolled consecutively if they met the inclusion criteria. Peripheral blood was obtained between day 10 and day 13 during the menstrual cycle.

For the study group (RIF group), the inclusion criteria were: (1) primary infertility; (2) between 25 and 45 years old, without active disease including autoimmune disease and not on any medication; (3) no history of ultrasound detectable embryos in uterus; (4) women with a history of two or more implantation failures undergoing IVF-ET cycles due to a defect of implantation (hCG negative) who had one or more morphologically good quality embryos transferred per each IVF cycle, excluding donor oocyte cycles; (5) no apparent cause for IVF failures could be documented; (6) at least two months after last failed IVF-ET cycle; (7) no male factor infertility; (8) no uterine anatomical abnormalities; (9) no tubal factor infertility; (10) no endometriosis; (11) no abnormal thyroid function or prolactin level; (12) regular menstruation; (13) no any positive test of antinuclear antibody, antithyroglobulin, antimicrosomal antibodies, lupus anticoagulant or anticardiolipin antibodies; and (14) no active infection or history of malignancy.

For the 23 control group, the inclusion criteria were: (1) not pregnant; (2) between 25-45 years old; (3) normal fertile healthy women with a history of one or more normal deliveries; (4) no history of infertility,
complicated pregnancies or spontaneous abortions; (5) at least two months since last delivery; (6) regular menstruation; (7) no history of endometriosis, abnormal thyroid function, or abnormal prolactin level; (8) no history of immune disorder; and (9) no active infection or history of malignancy.

Measurement of serum IL-17, IL-6, IL-23, TGF-β and IL-10 by ELISA

The serum levels of IL-17, IL-6, IL-23, TGF-β and IL-10 were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (IL-17 and IL-10 ELISA kits, both from Biosource, Nivelles, Belgium; IL-23 ELISA kit, from Bender MedSystems, Burlingame, CA, USA; IL-6 and TGF-β ELISA kits, both from R&D Systems, Minneapolis, MN, USA). The minimal detectable concentrations were 2 pg/mL for IL-17, 0.7 pg/mL for IL-6, 8 pg/mL for IL-23, 5 pg/mL for TGF-β and 7.8 pg/mL for IL-10. Intra-assay and inter-assay coefficients of variation for all ELISAs were <5% and <10%, respectively. All samples were measured in duplicate.

Statistical methods

When a cytokine or hormone was undetectable, we used the minimum detectable concentration to represent its value. Results are presented as the median (interquartile range) unless specified otherwise. The Mann-Whitney U test was used to compare variables between groups. The correlations between variables were analyzed using Spearman’s rank correlation coefficient. A probability of less than 0.05 was considered significant. Statistical calculations were performed using the Statistical Package for the Social Sciences (SPSS), Windows Version 13.0 (SPSS Inc., Chicago, IL, USA).

Results

Demographic data and clinical characteristics of women with RIF and controls

Twenty-eight women were enrolled in the RIF group and 23 women were enrolled in the control group. The demographic data and number of previous failed IVF-ET cycles were summarized in Table 1. There were no significant differences in age and number of IVF-ET cycle between groups.

Comparisons of serum cytokines between women with RIF and controls

The data on serum IL-17, IL-6, IL-23, IL-10 and TGF-β are summarized in Table 1. The level of serum IL-10 was significantly higher in women with RIF than that in controls (median = 28.83 pg/mL, interquartile [IQ] range 20.70-83.14 pg/mL vs. median = 14.48 pg/mL, IQ range 11.81-26.98 pg/mL, p=0.006).

Comparisons of Th17/Treg cytokine ratios between women with RIF and controls

The IL-17/IL-10 ratio was significantly lower in women with RIF than that in controls (median = 0.07, IQ range 0.02-0.17 vs. median = 0.04, IQ range 0.01-0.09, p=0.047). The IL-23/IL-10 ratio was also significantly

Table 1. Demographic data, clinical characteristics and laboratory findings in women with normal fertility and in those with repeated implantation failures (RIF) after in vitro fertilization and embryo transfer (IVF-ET) cyclesa

<table>
<thead>
<tr>
<th></th>
<th>Control (n = 23)</th>
<th>RIF (n = 28)</th>
<th>Total (n = 51)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>37.0 (35.0-42.0)</td>
<td>36.0 (34.0-39.0)</td>
<td>37.0 (34.0-41.0)</td>
</tr>
<tr>
<td>No of IVF-ET cycles</td>
<td>0</td>
<td>3.0 (2.0-4.0)</td>
<td>-</td>
</tr>
<tr>
<td>IL-17 (pg/mL)</td>
<td>18.61 (14.79-35.02)</td>
<td>20.18 (7.52-31.54)</td>
<td>20.11 (13.30-33.17)</td>
</tr>
<tr>
<td>IL-6 (pg/mL)</td>
<td>12.14 (8.04-22.68)</td>
<td>9.95 (7.12-20.63)</td>
<td>10.61 (7.80-22.12)</td>
</tr>
<tr>
<td>IL-23 (pg/mL)</td>
<td>95.81 (83.55-107.75)</td>
<td>92.71 (16.28-110.88)</td>
<td>93.74 (56.67-109.54)</td>
</tr>
<tr>
<td>IL-10 (pg/mL)</td>
<td>14.48 (11.81-26.98)</td>
<td>28.83 (20.70-83.14)</td>
<td>23.69 (12.80-42.62)</td>
</tr>
<tr>
<td>TGF-β (pg/mL)</td>
<td>358.50 (260.91-465.46)</td>
<td>430.93 (172.81-722.56)</td>
<td>369.17 (251.09-583.49)</td>
</tr>
<tr>
<td>IL-17/IL-10 (ratio)</td>
<td>1.19 (0.63-2.47)</td>
<td>0.39 (0.14-1.66)</td>
<td>0.87 (0.18-2.39)</td>
</tr>
<tr>
<td>IL-17/ TGF-β (ratio)</td>
<td>0.055 (0.037-0.061)</td>
<td>0.044 (0.028-0.100)</td>
<td>0.049 (0.035-0.086)</td>
</tr>
<tr>
<td>IL-23/IL-10 (ratio)</td>
<td>6.20 (3.33-9.18)</td>
<td>1.27 (0.37-4.92)</td>
<td>4.37 (0.54-6.69)</td>
</tr>
<tr>
<td>IL-23/ TGF-β (ratio)</td>
<td>0.25 (0.18-0.36)</td>
<td>0.16 (0.10-0.26)</td>
<td>0.20 (0.11-0.29)</td>
</tr>
<tr>
<td>IL-6/IL-10 (ratio)</td>
<td>0.82 (0.46-1.36)</td>
<td>0.52 (0.13-0.81)</td>
<td>0.62 (0.22-1.02)</td>
</tr>
<tr>
<td>IL-6/ TGF-β (ratio)</td>
<td>0.036 (0.020-0.081)</td>
<td>0.031 (0.017-0.051)</td>
<td>0.035 (0.018-0.060)</td>
</tr>
</tbody>
</table>

aValues are median (interquartile range) unless designated otherwise.
bp<0.01 compared with control
cp<0.05
lower in women with RIF than in controls (median = 11.2, IQ range 1.0-25.7 vs. median = 2.9, IQ range 1.0-19.3, p=0.002). In addition, the IL-23/TGF-β ratio was significantly lower in women with RIF than that in controls (median = 0.16, IQ range 0.10-0.26 vs. median = 0.25, IQ range 0.18-0.36, p=0.031); and the IL-6/IL-10 ratio was significantly lower in women with RIF than that in controls (p=0.008).

**Correlations between Th17 and Treg related cytokines**

For all subjects, IL-17 level had a significant positive correlation with IL-23 level (Fig. 1A). IL-17 had significant negative correlations with IL-10 (Fig. 1B). But IL-23 had a significant positive correlation with IL-10 (Fig. 1C). There were no significant correlations among other cytokines.

**Discussion**

To our knowledge, this is the first study to compare the serum Th17 related cytokines (IL-17, IL-6, IL-23) and Treg related cytokines (IL-10, TGF-β) and their ratios between non-pregnant women who had RIF with negative hCG after IVF and those with normal fertility. Cheng et al. raised the notion of a Th17/Treg balance and reported that there was an imbalance in patients with acute coronary syndrome, an inflammatory disease [13]. The positive correlation between IL-17 and IL-23 and the negative correlation between IL-10 and IL-17 found in our subjects supported the Th17/Treg balance hypothesis. The lower level of IL-10 and lower ratio of Th17/Treg related cytokines (IL-17/IL-10, IL-23/IL10, IL-23/TGF-β, IL-6/IL-10) in women who had RIF with negative hCG after IVF in late follicular phase might suggest a pre-existing skewed Treg cytokine response. However, the role and value of Th17/Treg related cytokines needs further study. It is important to investigate serial serum Th17/Treg related cytokines prior to an IVF cycle and peri-implantation period and then correlate them with the pregnancy outcome. The data may help to elucidate whether a pre-existing skewed Treg cytokine response, which might cause inadequate inflammation in the endometrium during early implantation, is present and related to the defect of implantation. In addition, serum cytokine levels can not reflect true situations in endometrium. Therefore, concomitant investigation of serum and endometrial Th17/Treg functions on different levels, including cell frequencies and key transcription factors, is necessary to

![Figure 1. Correlations between serum concentrations of (A) IL-23 and IL-17, (B) IL-10 and IL-17, and (C) IL-10 and IL-23 for all subjects. Data indicate positive associations in (A) and (C) and a negative association in (B). Data were analyzed by Spearman’s rank correlation coefficient.](image-url)
Th17/Treg-related cytokines in implantation failure

elucidate the role of Th17/Treg balance in implantation defect.

In conclusion, our results suggest decreased ratios of serum Th17/Treg related cytokines may play a role in the pathogenesis of a defect resulting in implantation failure. However, this study did not address a direct cause and effect relationship between maternal serum Th17/Treg imbalance and an implantation defect. The biological significance of the imbalance of serum Th17/Treg-related cytokines remains to be evaluated by larger prospective studies.

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References

在反覆性經體外受精及胚胎植入後因著床缺陷而導致著床失敗之女性血清中Th17/Treg相關細胞激素比值較低

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目的：比較反覆性經體外受精及胚胎植入後因為著床缺陷（人類絨毛膜性腺激素陰性）而導致著床失敗之女性及具有正常生育能力（對照組）之女性之間，血清中之第17型輔助T細胞/調節性T細胞相關之細胞激素比值是否有差異。方法：使用酵素免疫分析法來測量28位反覆著床失敗之女性及23位具有正常生育能力之女性血清中之介白質17，介白質23，介白質6，介白質10及移轉生長因子-β之濃度。結果：反覆著床失敗之女性之血清中第17型輔助T細胞相關之細胞激素/調節性T細胞相關之細胞激素之比值，包括介白質17/介白質10，介白質23/介白質10，介白質23/移轉生長因子-β，介白質6/介白質10，皆顯著地比正常對照組女性低。結論：血清中第17型輔助T細胞/調節性T細胞相關之細胞激素比值較低可能在著床缺陷之致病機轉上扮演一個角色。

關鍵詞：第17型輔助T細胞，調節性T細胞，細胞激素，著床失敗