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Research Paper: Are There Any Differences Between the Distribution of Placental Bed Leukocyte Subtypes and Plasma Cytokine Levels of Preeclamptic and Healthy Pregnants?

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ABSTRACT

Objectives: Preeclampsia (PE) is associated with impaired decidual leukocyte and plasma cytokine balance compared with normal pregnancy. We aimed to investigate maternal plasma levels of Interferon-gamma (IFN-g), Tumor Necrosis Factor-alpha (TNF-a), Transforming Growth Factor-beta (TGF-b), Interleukin-4 (IL4), IL6, IL10, IL17, IL35, suppressor of Cytokine Signalling-3 (SOCS3) and placental bed leukocytes in preeclamptic and healthy pregnants.

Materials & Methods: This study was conducted with 40 preeclamptic and 40 normotensive pregnants. Cytokine levels were studied with enzyme-linked immunosorbent assay. CD8, CD56 and CD163 antigens were analysed by immunohistochemical study on placental bed biopsies.

Results: In preeclamptic women; IFN-g and TGF-b levels were significantly higher and IL-35 levels were significantly lower than those of controls. CD8, CD56 and CD163 positivity of preeclamptic group were not significantly higher than those of controls. CD8 staining showed negative correlation with plasma IL17 levels. CD163 staining showed negative correlation with TNF-a/IL4 ratio. TNF-a/IL4 ratio showed minimal influence on placental bed CD163 staining.

Conclusion: Slightly increased placental bed CD8, CD56 and CD163 positive leukocytes and increased plasma IFN-g, TGF-b and decreased plasma IL35 levels of preeclamptic pregnants indicate an aberrant cell mediated immunity in PE. We could not say yet that this condition is whether result or reason. New studies are needed to discuss our results.

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1. Introduction

Preeclampsia (PE) is still a leading cause of maternal and fetal morbidity and mortality. Despite decades of intense research on the problem, there is no early predictive test to recognize those at risk. Recently, altered immune responses have been suspected to be involved in PE pathogenesis [1]. During pregnancy, the balance of T helper1 (Th1) (cell-mediated immunity) and Th2 (humoral immunity) cytokines is characterized by an initial prevalence of Th2 cytokines, followed by a progressive shift toward Th1 predominance late in gestation. Interferon-gamma (IFN-g) and tumour necrosis factor-alpha (TNF-a) are the cytokines of Th1, interleukin-4 (IL4), IL6 and IL10 are the cytokines of Th2. The abnormality in Th1/Th2 balance may initiate and intensify the cascade of inflammatory cytokine production involved in adverse pregnancy outcomes as spontaneous abortion, intrauterine growth restriction, PE and preterm delivery [2-4]. Besides the imbalance of Th1 and Th2 cells, alterations of the prevalence of Th17, regulatory T (Treg) cells, cytokine response systems (suppressor of cytokine signalling-SOCS) have also been suggested to contribute to pathogenesis of PE [5-13].

Immigrant and resident leukocytes are the main immune components of decidual tissue [14, 15]. 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 [14, 16-19]. It was reported that decidual macrophages with CD163 marker play important role on immune regulation via inhibition of autologous T-cells proliferation [20]. Some investigators reported decreased T lymphocytes and macrophages in the third trimester placental bed biopsies of preeclamptic women [21]. But another researchers indicated increment of macrophages in the first trimester decidua of preeclamptic women [22].

In this study, we researched the hypothesis that PE is associated with impaired decidual leukocyte and plasma cytokine balance compared with normal pregnancy; and for this hypothesis we evaluated the maternal plasma levels of IFN-g, TNF-a, transforming growth factor-beta (TGF-b), IL4, IL6, IL10, IL17, IL35, SOCS3 and placental bed leukocyte subpopulations in preeclamptic and healthy pregnants.

2. Materials and Methods

This study was conducted with 40 preeclamptic (study group) and 40 normotensive pregnant (control) women in third trimester at Firat University Hospital, Department of Obstetrics and Gynecology, after local ethical committee approval between August 2011 and August 2012. Normal pregnant women who experienced elective cesarean section were recruited when they were admitted to delivery unit. Normal pregnancy was defined as pregnancy with normal blood pressure (<140/90 mmHg), no proteinuria, and absence of obstetric and medical complications. Women diagnosed with PE were recruited when they were admitted to delivery unit. Diagnosis of PE was defined as follows: sustained systolic blood pressure of >140 mmHg or a sustained diastolic blood pressure of >90 mmHg on two separate readings; proteinuria measurement of 1+ or more on dipstick, or 24-h urine protein collection with >300 mg in the specimen. Proteinuria severity was defined according to the grading of dipstick. The pregnants on active labor, smokers and patients with signs of infection were excluded. To avoid clinical phenotypic differences in preeclamptic patients, patients complicated with HELLP syndrome (Hemolysis, Elevated Liver enzyme and Low Platelet count), diabetes, and/or renal disease were excluded. Signed consent was obtained at the time of enrollment.

ELISA assay

Maternal prepartum venous blood samples were drawn during transfer to cesarean section using a polypropylene syringe and a butterfly needle and then 7 mL blood was transferred to tubes. The samples were centrifuged at 2500 rpm at 4°C for 15 min, 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 IL17, IFN-g, TNF-a, TGF-b, IL6, IL4, IL10 (Boster Biological Technology, Fremont, CA) and SOCS3 and IL35 (USCN, Wuhan, China) according to the manufacturer's instructions. The samples were analyzed 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.

Immunohistochemical study

Placental bed biopsies were taken after peeling off the placenta from the uterine wall under direct vision during cesarean section. A fragment of decidua and underlying myometrium of approximately 1.5 cm in diameter was removed using a scalpel and a scissor. The biopsies were taken centrally which was determined manually before removal of placenta. Tissues were fixed in the neutral 10% formaldehyde, embedded paraffin, cut in 5-mm sections and stained with Hematoxilen-Eosin. For immunohistochemical staining, 5 mm paraffin sec-

tions 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 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 counterstained 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.

Statistical analysis

Statistical analysis was performed by Statistical Package for Social Sciences 16.0 (SPSS Inc., Chicago, IL) version. Results were presented as mean±SE. Differences in continuous variables were analyzed by Student's t-test or Mann–Whitney U-test according to distribution of data. Differences between groups for categorical variables were analyzed using the chi-square test or Fisher's exact test, as appropriate. The relations among plasma cytokine levels, placental bed leukocyte subpopulations and clinical characteristics were evaluated by Spearman correlation test. Logistic regression analysis was employed to identify the cytokines and leukocyte subtypes which could have influenced on blood pressure, proteinuria, birthweight and Apgar1 score. $P<0.05$ were considered as statistically significant.

3. Results

Demographic and clinical characteristics of all women in the study were presented in Table 2. Gestational week, birth-weight and apgar1 score of preeclamptic women were lower than of controls. $MgSO_4$ treatment was applied to 67.6% of preeclamptic women.

Cytokine levels of all women in the study were presented in Table 3. In preeclamptic women; IFN-g and TGF-b levels were significantly higher ($P<0.01$) and IL-35 levels were significantly lower ($P<0.01$) than those of controls. The comparison of Th1 and Th2 cytokine ratios in two groups revealed out the following findings: IFN-g/IL-10, IFN-g/IL-6, and IFN-g/IL-4 ratios of PE group were significantly higher than those of control group ($P<0.01$). IL-35/IL17 ratio was significantly low in PE group compared to that in control group ($P<0.01$). There was no significant difference between groups for the ratios of TNF-alpha/IL-10, TNF-alpha/IL-6 and TNF-alpha/IL-4.

Immunohistochemical staining results of CD8, CD56 and CD163 antigens on placental bed biopsies were presented in Table 4. The staining dominancy of leukocyte subpopulations on placental bed biopsies was observed for CD163 and CD8 antigens. Staining degree of CD56 was lower than those of CD163 and CD8. Staining degree of CD8, CD56 and CD163 positive cells in preeclamptic group were higher than those of control group; but the differences were not significant.

In correlation analysis; leukocyte subtype stainings showed no relation with blood pressure, proteinuria severity, apgar1 score and birthweight. CD56 and CD163 showed no relation with all cytokines. CD8 staining showed negative correlation with plasma IL17 levels ($R=0.28$, $P=0.027$). CD163 staining showed negative correlation with TNF-a/IL4 ratio ($R=0.29$, $P=0.025$). While IFN-g and TGF-b levels showed positive correlation with blood pressure; IL17, IL35 and SOCS3 levels showed negative correlation with blood pressure.

In regression analysis, plasma IL17 levels showed no influence on placental bed CD8 staining, but TNF-a/IL4 ratio showed minimal influence on placental bed CD163 staining (OR=0.3, 95% CI=0.58-8.48, $P=0.03$). After adjusting for gestational age, maternal age and proteinuria;

Table 1. Details of immunohistochemical analysis

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

Table 2. Demographic and clinical characteristics of all women in the study

Parameters	Preeclampsia (n=40)	Control (n=40)	P
Age (years)	31.1±1	32.1±0.8	0.44
Gestational week	35(24-40)	38(34-40)	<0.01[a]
Hemoglobin (gr/dL)	12.2(7-16)	11.6(8.1-14.4)	0.15[a]
Plateletx1000 (/mm^3)	238±15	263±15	0.24
ALT (IU/L)	18.5(8-40)	17(6-32)	0.03[a]
AST (IU/L)	30(16-45)	23(11-38)	0.01[a]
Birth weight (gr)	2007(510-3900)	3170(2014-3900)	<0.01[a]
Apgar1 score	8(4-9)	9(5-10)	0.04[a]

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Values are presented as mean±SE and median (min-max). ALT: alanine aminotransferase; AST: aspartate aminotransferase; [a]: Mann Whitney U test

none of the cytokines showed influence on blood pressure. After adjusting for maternal age, gestational age and blood pressure; none of the cytokines showed influence on proteinuria severity. After adjusting for maternal age, gestational age, proteinuria and blood pressure; none of the cytokines showed influence on birth weight and Apgar1 score.

4. Discussion

In this study, we observed statistically not significant increament of placental bed CD8 (cytotoxic T lymphocyte), CD56 (natural killer cell) and CD163 (macrophage) antigen staining in preeclamptic pregnants compared to those of healty pregnants. We also demonstrated that: 1) TNF-a/IL4 ratio showed minimal influence on placental bed CD163 staining; 2) IFN-g/IL-10, IFN-g/IL-6, and IFN-g/IL-4 ratios of PE group were significantly higher than of control group; 3) IL-35/IL17 ratio was significantly low in PE group compared to that in control group; 4) after adjusting for gestational age, maternal age and proteinuria; none of the cytokines showed influence on blood pressure; 5) after adjusting for maternal age, gestational age and blood pressure; none of the cytokines showed influence on proteinuria severity. The limitations in our study were as follows; firstly our con-

Table 3. Plasma cytokine levels of all women in the study

Cytokines	Preeclampsia (n=40)	Control (n=40)	P
IL-17 (ng/mL)	1.28(1.08-4.24)	1.5(1-3.57)	0.44[a]
IL-6 (ng/mL)	0.31(0.01-8.2)	0.34(0.01-6)	0.29[a]
IL-4 (ng/mL)	1.24(0.03-30.97)	1.5(0-11)	0.75[a]
IL-10 (ng/mL)	9.81(8.58-10.04)	9.65(6.15-10.04)	0.21[a]
IL-35 (ng/mL)	6.65(3.7-1000)	15.3(3.5-987)	<0.01[a]
SOCS-3 (ng/mL)	63.09(4.2-703)	82.05(5-714)	0.08[a]
IFN-gamma (ng/mL)	0.42(0-27.3)	0(0-5.86)	<0.01[a]
TGF-beta (ng/mL)	2.6(1.4-7.6)	1.9(0.4-15.2)	<0.01[a]
TNF-alpha (ng/mL)	0.65(0.3-10.3)	0.8(0.09-62.2)	0.17[a]

Values are presented as median (min-max). [a]: Mann Whitney U test

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Table 4. Immunohistochemical study of placental bed biopsies in all women

Antigen	Preeclampsia[a] (n=40)	Control[a] (n=40)	P
CD163	36.92±3.92	43.56±5.23	0.32
CD56	7.75±3.36	15.43±5.86	0.22
CD8	41.36±5.57	44.69±7.19	0.71

[a]: Mean number and standard error of positive cells in 3 random fields (320x)

trol group were not consisted of gestational age-matched healthy pregnants. Secondly, we did not know the flow-cytometric leukocyte subtypes of pregnants.

The mechanism for increased inflammatory response in PE is largely unknown. The evaluation of 31 preeclamptic and 67 normotensive maternal plasma samples for the parameters of IL4, IL10, and IFN-g revealed out no difference; but an association between PE and increased TNF-a and IL6 levels were noticed [23]. Other researchers reported high serum TNF-a and IFN-g levels in 34 preeclamptic women compared to 16 healthy pregnant women [24]. In an animal study, researchers determined that TNF-a supression ameliorated Th1-induced PE-like symptoms in mice [25].

In an another study, a significant difference between serum levels of preeclamptic and normotensive pregnants for parameters of IL4, IL6 and IL10 was observed [26]. In our study we observed significantly high IFN-g and TGF-b levels in preeclamptic pregnants compared to those in healty pregnants. Increased IFN-g/IL10, IFN-g/IL6, IFN-g/IL4 ratios in our population pointed out the impaired Th1/Th2 balance in PE. Dhillion et al. observed increament of blood pressure after IL-17 infusion on pregnant rats [27]. Increased IL-17 producing T lymphocytes were observed in preeclamptic pregnants [6].

Toldi et al. demonstrated increased IL-17 expression in PE [5]. A novel inhibitory cytokine, IL-35 is produced by Foxp3(+) regulatory T-cells (Tregs) and mediates maximal suppression of Tregs. First-trimester human trophoblasT-cells expressed and secreted IL-35, which might contribute to suppressive capacity of maternal immune cells. IL-35 may be an important factor of the cytokine network regulating local immune responses during human pregnancy [28]. IL-35 is also an anti-inflammatory cytokine suppressing the immune response through the expansion of Tregs and suppression of Th17 cell development [29, 30]. In our study we observed significantly low IL-35 levels and decreased IL-35/IL-17 ratio in PE and this condition may refer to inflammatory status in PE.

Decidual leukocyte adaptations during pregnancy contribute to trophoblast invasion and spiral artery remodeling. Dysregulation of the interaction among macrophage, NK and T-cells may lead to PE [31-33]. According to study of Lockwood et al. excess macrophage-derived TNF-a production interferes with normal stepwise spiral artery remodelling and leading to PE. But decidual NK cell-derived IFN-g reverses effect of TNF-a in human decidual cells and protects against PE [34]. In our study we observed significantly increased plasma IFN-g levels and IFN-g/IL10, IFN-g/IL6 and IFN-g/IL4 ratios in preeclamptic women. These results might be the compensatory study of immune system for protection against PE. Women with pregnancy-related disorders show dysregulation in IFN-g, and decidual NK cells [35].

Williams et al. reported decreased CD56 and CD8 positive cells on decidua of preeclamptic pregnants compared with third trimester controls. But percentage of each of the leukocyte subtype did not differ between PE and controls [21]. Sasaki et al. suggested that accumulation of CD8+T-cells at the placental bed in PE might reverse maternal tolerance, resulting in fetal rejection [36]. In an another study it was stated that preeclamptic decidua showed decreased CD8+T-cells and CD56+NK cells compared to those in normal pregnancy [37]. Wong et al. observed higher incidence of hypertensive pregnancy disease in women with increased NK cells on peri-implantation period endometrium than in women with normal NK cells count [38]. Other researchers showed significantly decreased NK cell numbers in preeclamptic decidua compared with gestational age-matched controls [39].

In our study we observed slightly increased, but not significant, CD56 and CD8 staining on preeclamptic decidua compared with normotensive decidua. In an expression study, investigators reported decreased CD163 level on placenta of preeclamptic patients compared with that of normotensive controls [40]. Another researchers pointed out decreased CD163 expression on monocyte surface of preeclamptic pregnants compared with normotensive pregnants [41].

Kronborg et al. observed no difference between preeclamptic and normotensine pregnants for parameter of soluble macrophage serum marker sCD163 [42]. Schonkeren et al. reported significantly increased CD163 expression in preterm preeclamptic decidua basalis compared with preterm control pregnancies [43]. This difference from other studies may arise from their preterm study population. In our study CD163 staining on placental bed biopsies of preeclamptic pregnants were slightly higher than of normotensive controls. Also plasma TNF-a/IL4 ratio showed negative correlation with CD163 staining and this correlation brought minimal influence on placental bed CD163 staining. And in our opinion impaired antiinflammatory status due to this interaction may lead to PE. Of course we could not know whether this condition is reason or result.

5. Conclusion

In this study we observed increased Th1 type cytokines and decreased IL35 levels on plasma of preeclamptic pregnants. Slightly increased placental bed leukocytes with surface antigen of CD8, CD56 and CD163 did not show concordance with other studies. This diversity may arise from our ethnicity difference. Both of increased Th1 type cytokines and increased leukocytes indicate an aberrant cell mediated immunity in PE. New studies are needed to discuss our results.

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Conflict of Interest

The authors declared no conflicts of interest.

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