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Early pregnancy immune biomarkers in peripheral blood may predict preeclampsia

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ABSTRACT

We performed a prospective cohort study in 197 pregnant women. Peripheral blood was collected between 5 and 16 weeks of gestation. Intracellular cytokine analysis and immunophenotype were performed by flow-cytometry. Serum levels of cytokines and chemokines were analyzed by multiplex assay. 86 patients were eligible for the analysis and 10.5% (n = 9) developed preeclampsia. Patients with preeclampsia had significantly higher percentage of $CD3^+CD4^+TNF\alpha^+$ T helper (Th) 1 cells (45.4 ± 10.3 vs 37.1 ± 8.5, $P = 0.032$) and $CD3^+CD4^+IL17^+$ Th 17 cells (2.4 ± 1.3 vs 1.6 ± 1.1, $P = 0.029$) when compared to those of patients without preeclampsia. $CD3^+CD4^+CD25^+CD127^{dim/-}$ T regulatory cells (Treg) cells (5.7 ± 1.2% vs 7.0 ± 1.6%, $P = 0.015$) were significantly lower in patients with preeclampsia when compared to those without preeclampsia. Patients with preeclampsia had significantly higher TNFα/IL-10 cell ratio (43.8 ± 10.3 vs 34.3 ± 7.9, $P = 0.005$) and Th17/Treg cell ratio (0.5 ± 0.3 vs 0.2 ± 0.2, $P = 0.011$) when compared to those of patients without preeclampsia. IL-8 and Macrophage inflammatory protein (MIP)-1α serum levels were significantly higher in patients with preeclampsia when compared with patients without preeclampsia (Median = 341.0 vs 87.6, U = 152, $P = 0.020$ and Median = 35.7 vs 17.7, U = 120, $P = 0.029$ respectively). Serum MCP-1 levels were significantly lower in patients with preeclampsia when compared with patients without preeclampsia (Median = 233.8 vs 390.9, U = 183, $P = 0.021$). The logistic regression predictive model combining TNFα/IL-10 ratios, IL-8 and MCP-1 serum levels had the best performance (AUC = 0.886, 95%CI 0.8–0.9). We concluded that elevated Th1 and Th17 cell percentages, elevated TNFα/IL-10 and Th17/Treg cell ratios and decreased Treg cell percentages in early pregnancy are associated with preeclampsia.

1. Introduction

Preeclampsia is a multisystem disorder characterized by new onset hypertension with coexisting proteinuria, other maternal organ dysfunction such as renal dysfunction, impaired liver function, neurological or hematological complications, and/or uteroplacental dysfunction after 20 weeks of gestation (Mol et al., 2016; Tranquilli et al., 2014; Chaiworapongsa et al., 2014). This gestation-specific syndrome affects 2–8% of all pregnancies, and is a leading cause of maternal and perinatal morbidity and mortality (Ananth et al., 2013).

The major pathogenesis of preeclampsia is thought to result from an abnormal placenta. Incomplete invasion of the cytotrophoblast fails to adopt an invasive endothelial phenotype with shallow invasion of small-caliber and resistant spiral arteries. The resultant ischemic placenta releases factors into the maternal circulation that are capable of inducing the clinical manifestations of the disease (Chaiworapongsa et al., 2014; Powe et al., 2011; Wang et al., 2009; Roberts and Bell, 2013). Nonetheless, it is still poorly understood what is the mechanism that leads to this poor placentation. Researchers have suggested that immunological alterations in the early placental microenvironment

Abbreviations: CCL11, C-C motif chemokine; EGF, epidermal growth factor; FoxP3, forkhead box P3; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HELLP, hemolysis elevated liver enzymes and low platelets; MIP, macrophage inflammatory protein; mAbs, monoclonal antibodies; ROC, receiver operating characteristic; RORc, retinoic acid-related orphan receptor C; T-bet, T cell box transcriptor factor; Th, T helper; TNF, tumor necrosis factor; Treg, T regulatory cells; VEGF, vascular endothelial growth factor

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may participate in the induction of preeclampsia.

Normal pregnancy has a distinct shift toward a Th2 immuno-tolerant state in the peripheral circulation and at the fetal-maternal interface except at the time of implantation and parturition (Lin et al., 1993; Saito et al., 1999a). Preeclampsia has been characterized by Th1 predominance with pro-inflammatory state and Th1/Th2 imbalance was implicated as the main cause of preeclampsia (Saito and Sakai, 2003; Saito et al., 1999a). Additionally, Th17/Treg imbalance with up-regulated Th17 cells and downregulated Treg cells has been reported in women with preeclampsia and other obstetrical complications (Darmochwal-Kolarz et al., 2012; Rahimzadeh et al., 2016; Lau et al., 2013; Szarka et al., 2010). Based on these findings, preeclampsia is associated with an overall pro-inflammatory systemic environment. Elevated levels of pro-inflammatory cytokines, chemokines and adhesion molecules in the maternal circulation might play a central role in the excessive systemic inflammatory response, as well as in the generalized endothelial dysfunction, which is the major characteristic of the disease (Lau et al., 2013; Szarka et al., 2010).

Considerable efforts have been made to identify biomarkers to predict and apply as a therapeutic target for preeclampsia. Current strategies for first trimester prediction of preeclampsia are based in the combination of baseline maternal factors, biophysical parameters and placenta related proteins (Forest et al., 2012; Poon and Nicolaides, 2014; Park et al., 2015). The combination of these markers can identify early-preeclampsia but unfortunately they have poor performance for detecting all cases of preeclampsia (Pedrosa and Matias, 2011). Considering the compiled evidences that the possible underlying immunopathology of preeclampsia routed from abnormal angiogenesis and implantation, investigation of immune responses during the first trimester may shed a light to develop a biomarker that can predict preeclampsia much earlier. Early detection of high-risk pregnant women for preeclampsia may open a possible option for primary prophylactic measures. Therefore, we investigated the peripheral blood immune effectors in normal and preeclamptic women and evaluated their use as predictors of preeclampsia in early gestations.

2. Materials and methods

2.1. Population

We performed a prospective cohort study from March 2014 to July 2014. Study candidates were enrolled sequentially at Department of Obstetrics and Gynecology, Mount Sinai Hospital Medical Center, Chicago, IL, USA. The study was approved by the Institutional Review Board and all patients signed an informed consent prior to entering the study. Women with ongoing pregnancy of < 16 weeks of gestation and with maternal age between 18–40 years old were included. During the study period, a total of 197 pregnant women were recruited. Out of 197 women, 104 patients had lost follow-up, 4 had pregnancy losses and 3 had missing laboratory tests. The final total of 86 patients were eligible for the analysis.

Peripheral blood was collected between 5 and 16 weeks gestation (mean ± SD, 10.5 ± 2.6 weeks). Gestational age was calculated on the basis of last menstrual period and crown-rump length at the first trimester ultrasound. During pregnancy, women were treated according to the current practice guidelines of the American College of Obstetrics and Gynecology and no treatment modifications were performed based on the results of our study. Patients were followed up until delivery and clinical data were collected.

2.2. Flow cytometry

To characterize lymphocyte subsets, peripheral blood was collected in tubes with sodium heparin and stained with monoclonal antibodies against cell surface markers. Blood was incubated with antibody cocktails for 30 min at room temperature and then diluted in wash buffer. After one centrifugation, we used the combination of Immuno-Lyse and Fixative (Beckman Coulter) to wash red blood cells and fix white blood cells. After further washing, cells were analyzed using a FACSDiva flow cytometer (BD). For immunophenotyping we used the following mABs: KO-conjugated anti-CD45 (clone J.33), APC-A750-conjugated anti-CD3 (clone UCHT1), APC-conjugated anti-CD5 (clone BL1a), APC-conjugated anti-CD4 (clone 13B8.2), FITC-conjugated anti-CD8 (clone B9.11), PE-conjugated anti-CD28 (clone CD28.2), PE-conjugated anti-CD25 (clone 1HT44H3), PC7-conjugated anti-CD19 (clone J3-119), FITC-conjugated anti-CD16 (clone 3G8), and V450-conjugated anti-CD56 (clone B159) (Beckman Coulter). The number of events acquired for each sample was 10,000 lymphocytes. Lymphocytes were gated based on forward scatter and CD45 expression. We then calculated the percent of lymphocytes that were B cells ($CD3^-$ $CD19^+$), CD5+ B cells, NK cells ($CD3^-$ $CD56^+$), conventional cytotoxic NK cells ($CD3^-CD56^+$ $CD16^+$), T cells ($CD19^-$ $CD3^+$), $CD4^+$ T cells, $CD8^+$ T cells, $CD56^+$ T cells, $CD28^+$ T cells, or $CD25^+$ T cells. Flow cytometric analysis was applied to measure the percent of CD4+ T regulatory cells (Treg), defined as $CD25^{bright}$ $CD127^{dim/-}$ For Treg analysis, CD45 and CD3 mAB along with the BD Human Regulatory T Cell Cocktail were utilized: FITC-conjugated anti-CD4; PE-Cy7-conjugated anti-CD25; Alexa Fluor 647-conjugated anti-CD127.

2.3. Intracellular cytokine analysis

Intracellular cytokine analysis was performed as previously described. (Kwak-Kim et al., 2003) Briefly, freshly isolated PBMC were resuspended in 5×10^6/mL RPMI-1640 supplemented with 10% fetal calf serum and antibiotic-antimycotic solution. Then, PBMCs (5×10^5) were incubated for 16 h at 37 °C with 5% CO_2 in the presence of 25 ng/mL of phorbol myristate acetate (PMA), 1 μM of ionomycin (both from Sigma-Aldrich) and 0.7 μL of Brefeldin A (GolgiPlug, BD Biosciences) per 1 mL of cell suspension. After the incubation, the cells were washed and labeled with mAbs KO-conjugated anti-CD45 (clone J.33, Beckman Coulter), APC-H7-conjugated anti-CD3 (clone SK7, BD) and BV412-conjugated anti-CD8 (clone RPA-T8, BD). Cytofix/Cytoperm and Perm/Wash buffer (BD Pharmingen) were utilized to fix and permeabilize the cells according to manufacturer's instructions. To detect intracellular cytokines, the cells were stained with mAbs PE-conjugated anti-TNF-α (clone MAb11, BD), FITC-conjugated anti-IFN-γ (clone 45.15, Beckman Coulter), APC-conjugated anti-IL-10 (clone JES3-19F1, BD) and PE-Cy7-conjugated anti-IL-17 (clone BL168, BD).

Samples were analyzed using a FACSDiva flow cytometer (BD). T cells were gated based on CD45 and CD3 expression. $CD4^+$ cells were detected by negative selection ($CD3^+CD8^-$) within $CD45^+$ lymphocytes since CD4 is down-regulated by PMA/ionomycin stimulation. To calculate Th1/Th2 ratios, the percentage of $CD3^+CD4^+$ T cells expressing TNF-α or IFN-γ was divided by the percentage of $CD3^+CD4^+$ T cells expressing IL-10. To calculate the Th17/Treg ratio, the percentage of $CD3^+CD4^+$ cells expressing IL-17 were divided by the percentage of $CD4^+CD25^{bright}CD127^{dim/-}$ T cells.

2.4. Luminex assay

Peripheral blood was collected in serum separator tubes and spun within 2 h to isolate serum. Serum samples were frozen and stored at −80 °C until the analysis. Cytokines and chemokines, including EGF, G-CSF, GM-CSF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17, IP-10, MCP-1, MIP-1α, MIP-1β, TNF-α, TNF-β, VEGF and Eotaxin/CCL11 were measured with a multiplex assay kit (Milliplex MAP kit; Millipore) using a Luminex MAGPIX instrument according to the manufacturer's instructions. For values below the limit of detection threshold, we assigned values of the limit of detection divided by 2.