Immunological factors in preeclampsia

Ph.D. Thesis

Amrita Halmos, M.D.

Clinical Medicine Doctoral School
Semmelweis University

Supervisor: Attila Molvarec, M.D., Ph.D.

Official reviewers: Éva Varga, M.D., Ph.D.
Edina Wappler, M.D., Ph.D.

Head of the Final Examination Committee:
Ferenc Paulin, M.D., D.Sc.
Members of the Final Examination Committee:
Katalin Keltai, M.D., Ph.D.
Pál Siklós, M.D., C.Sc.

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Introduction

Preeclampsia is a severe complication of human pregnancy with a worldwide incidence of 2-10%. It is one of the leading causes of maternal, as well as perinatal morbidity and mortality, even in developed countries. Despite intensive research efforts, the etiology and pathogenesis of preeclampsia are not completely understood. Increasing evidence suggests that an excessive maternal systemic inflammatory response to pregnancy, which is supposed to be triggered by inflammatory stimuli derived from the hypoxic and oxidatively stressed placenta, plays a crucial role in the pathogenesis of the disease. The systemic inflammatory response involves a rise in number and activation of leukocytes with production of proteases and proinflammatory cytokines, as well as the activation of endothelial cells, platelets, the coagulation and complement systems and the production of acute phase proteins. The development of preeclampsia is influenced by both genetic and environmental risk factors, suggesting its multifactorial inheritance.

α_2-Heremans–Schmid (α_2-HS) glycoprotein (fetuin-A, AHSG), the human homolog of bovine fetuin, is an abundant plasma/serum protein synthesized predominantly by hepatocytes. It belongs to the cystatin superfamily of cysteine proteinase inhibitors. AHSG is one of the rare negative acute phase proteins, the synthesis of which is decreased in the liver during the acute phase response. The protein has diverse biological functions including the regulation of osteogenesis and bone resorption, the prevention of unwanted mineralization and the inhibition of insulin receptor autophosphorylation and tyrosine kinase activity. Additionally, it promotes phagocytosis and has also opsonic properties. Circulating AHSG levels decrease in several conditions characterized by tissue damage, infection, inflammation or malignancy.

An important feature of systemic inflammation in preeclampsia is the absence of Th2 skewness characteristic for normal pregnancy, and thus the predominance of a Th1-type immunity. Besides the imbalance of Th1 and Th2 cells, alterations of the prevalence of regulatory T cells have also been suggested to be of importance in the development of maternal systemic inflammation in preeclampsia. The population of regulatory T cells has been further grouped based on intracellular and cell surface markers. These Treg subgroups were shown to have distinct functional properties that

play different roles in the regulation of the inflammatory response. Recent studies showed that FoxP3, required for the development of Tregs, is specifically expressed in most CD4+ CD25+ T cells but also in a small part of CD4+ CD25- T cells. Furthermore, the ectopic expression of FoxP3 induced suppressive function in peripheral CD4+ CD25- T cells. These results demonstrate that FoxP3 is a key player in Tregs. The conventional CD4+ CD25high Treg subset might be further divided into activated (CD4+ CD25high FoxP3high) and resting (CD4+ CD25high FoxP3low) regulatory T cells. Both of these subtypes are suppressive, however, they differ in proliferation dynamics and responsiveness. Terminally differentiated activated Treg cells are more responsive, but die rapidly, whereas resting Tregs have limited responsiveness, but are able to proliferate and convert into activated Treg cells with elevated FoxP3 expression. In recent studies, a peak of Treg population was found during the second trimester of pregnancy, and a subsequent gradual decrease to levels slightly higher than non-pregnant levels was detected during the postpartum period. However, data concerning the alteration of circulating Treg frequency in preeclampsia are not fully in line.

The complement system is activated with increased terminal complex formation in the third trimester of normal human pregnancy, and further in preeclampsia, as was shown by the elevated amounts of activation markers in the systemic circulation. Ficolins are pattern recognition molecules of the innate immune system that bind to carbohydrate moieties present on the surface of microbial pathogens, apoptotic and necrotic cells. Trophoblast apoptosis is a feature of normal pregnancy with an increment in preeclampsia, complicated further by necrosis. Ficolins have been reported to bind to the trophoblast cells undergoing apoptosis in the preeclamptic placenta. They act through two distinct routes: initiate the lectin pathway of complement activation in concert with attached MBL-associated serine proteases (MASPs) and by a primitive opsonophagocytosis.

Objectives

1. Despite intensive research efforts, the etiology and pathogenesis of preeclampsia are not completely understood. Increasing evidence suggests that preeclampsia is characterized by an excessive maternal systemic inflammatory response with activation of both the innate and adaptive arms of the immune system, involving an acute phase reaction. During the acute phase response, serum levels of positive acute phase proteins increase, whereas those of negative acute phase proteins decrease. The purpose of our study was to determine serum concentrations of a negative acute phase protein (AHSG) and a positive acute phase protein (CRP) in preeclamptic and healthy pregnant women, to investigate the relationship of clinical characteristics and laboratory parameters of the patients to serum AHSG concentrations, and also to determine the diagnostic accuracy of AHSG measurements in preeclampsia.

2. Regulatory T cells play an important role in the development of pregnancy-specific immune tolerance. Decreased frequency as well as dysfunction of regulatory T cells may play a role in the activation of both the innate and adaptive arms of the immune system found in preeclampsia. Immunoregulatory functions differ between distinct Treg subsets. Thus we aimed to determine the peripheral frequency of the CD4+ CD25- FoxP3+ Treg subset and its correlation with the conventional CD4+ CD25high FoxP3+ Tregs in normal pregnancy and preeclampsia compared to non-pregnant women. We also examined the proportion of the activated CD4+ CD25high FoxP3high Treg subset within conventional Treg cells.

3. The excessive maternal systemic inflammatory response characteristic of preeclampsia involves the activation of the complement system. To study complement activation in preeclampsia, we measured circulating levels of ficolin-2 and ficolin-3 and also determined complement activation products (C4d, C3a, SC5b9), angiogenic factors (soluble fms-like tyrosine kinase-1, placental growth factor) and markers of endothelial activation (von Willebrand factor antigen), endothelial injury (fibronectin) and trophoblast debris (cell-free fetal DNA) and their relationship to circulating ficolin levels in non-pregnant, healthy pregnant and preeclamptic women.

Patients and methods

Study participants

Our studies were designed using a case-controlled approach. 93 preeclamptic and 127 healthy pregnant women were involved in our study of AHSG and CRP acute phase proteins, 60 preeclamptic patients, 60 healthy pregnant women and 59 healthy non-pregnant women were involved in the study of serum ficolin levels, and 20 preeclamptic patients, 20 healthy pregnant and 12 healthy non-pregnant women were involved in our study of the frequency of regulatory T cells. The study participants were enrolled in the First Department of Obstetrics and Gynecology and in the Department of Obstetrics and Gynecology of Kútvölgyi Clinical Center, at the Semmelweis University, Budapest, Hungary. All women were Caucasian and resided in the same geographic area in Hungary. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection and fetal congenital anomaly. The women were fasting, none of the pregnant women were in active labor, and none had rupture of membranes. The healthy non-pregnant women were in the early follicular phase of the menstrual cycle (between cycle days 3 and 5), and none of them received hormonal contraception.

Preeclampsia was defined by increased blood pressure (≥140 mmHg systolic or ≥90 mmHg diastolic on ≥2 occasions at least 6 hours apart) that occurred after 20 weeks of gestation in a woman with previously normal blood pressure, accompanied by proteinuria (≥0.3 g/24h or ≥1 + on dipstick in the absence of urinary tract infection). Blood pressure returned to normal by 12 weeks postpartum in each preeclamptic study patient. Preeclampsia was regarded as severe if any of the following criteria was present: blood pressure≥160 mmHg systolic or ≥110 mmHg diastolic, or proteinuria≥5 g/24h (or ≥3 + on dipstick). Pregnant women with eclampsia or HELLP syndrome (hemolysis, elevated liver enzymes, and low platelet count) were not enrolled in this study. Early onset of preeclampsia was defined as onset of the disease before 34 weeks of gestation (between 20 and 33 completed gestational weeks). Fetal growth restriction was diagnosed if the fetal birth weight was below the 10^{th} percentile for gestational age and gender, based on Hungarian birth weight percentiles.

The study protocol was approved by the Regional and Institutional Committee of Science and Research Ethics of the Semmelweis University, and written informed consent was obtained from each patient. The study was conducted in accordance with the Declaration of Helsinki.

Laboratory methods

Blood samples were obtained from an antecubital vein into plain, as well as ethylenediamine tetraacetic acid (EDTA)- or sodium citrate anticoagulated tubes, and then centrifuged at room temperature with a relative centrifugal force of 3000 *g* for 10 minutes. The aliquots of serum and plasma were stored at -80 °C until the analyses.

Serum CRP concentration was measured by particle enhanced immunoturbidimetric assay (Cobas Integra 800, Roche, Mannheim, Germany, Cat. No. 20764930). Human CRP agglutinated with latex particles coated with monoclonal anti-CRP antibodies. The precipitate was determined turbidimetrically at 552 nm.

Serum concentration of α_2-HS glycoprotein was determined by radial immunodiffusion method using Goat Anti-Human α_2-HS Glycoprotein IgG fraction (DiaSorin Inc., Stillwater, Minnesota, USA, Cat. No. 81931).

Peripheral blood mononuclear cells (PBMCs) were separated by a standard density gradient centrifugation (Ficoll Paque, Amersham Biosciences AB, Uppsala, Sweden, 27 minutes, 400 *g*, 22 °C) from freshly drawn blood collected in lithium heparin-treated tubes (BD Vacutainer, BD Biosciences, San Jose, CA, USA). This cell suspension was washed twice in phosphate-buffered saline. Cells were suspended in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO, USA).

PBMCs were stained for 30 min at 4 ºC with PE Cy7-conjugated CD4 and APC-conjugated CD25 mAbs (PharMingen, San Diego, CA, USA). After washing, cells were fixed with Fixation/Permeabilization solution and treated with Permeabilization Buffer according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). They were then stained with PE-conjugated FoxP3 mAb (eBioscience) for 30 min at 4 ºC. After washing, cells were analyzed on a BD FACSAria flow cytometer (BD Biosciences). 200000 cells were recorded. The population of lymphocytes was gated

from PBMCs according to Forward Scatter Characteristics and Side Scatter Characteristics.

Plasma levels of ficolin-2 and ficolin-3 were measured by enzyme-linked immunosorbent assay (Hycult Biotech, Uden, the Netherlands, Cat. No. HK336 and HK340, respectively) on an automated ELISA analyzer (Elisys UNO, Human GmBH, Wiesbaden, Germany), according to the manufacturer's instructions. Levels of C4d, C3a and SC5b9 in maternal plasma were assessed with Quidel ELISA kits (San Diego, California, USA, Cat. No. A008, A015 and A029, respectively).

Standard laboratory parameters (clinical chemistry) were determined by an autoanalyzer (Cobas Integra 800, Roche, Mannheim, Germany) using the manufacturer's kits. Plasma von Willebrand factor antigen (VWF:Ag) levels were quantified by ELISA (Dakopatts, Glostrup, Denmark), while plasma fibronectin concentration by nephelometry (Dade Behring, Marburg, Germany), according to the manufacturer's instructions.

Serum total soluble fms-like tyrosine kinase-1 (sFlt-1) and biologically active placental growth factor (PlGF) levels were measured by electrochemiluminescence immunoassay (Elecsys, Roche, Mannheim, Germany, Cat. No. 05109523 and 05144671, respectively) on a Cobas e 411 analyzer (Roche, Mannheim, Germany).

After extracting DNA with the silica adsorption method, the amount of cell-free fetal DNA in maternal plasma was determined in patients with male newborns by quantitative real-time PCR analysis of the sex-determining region Y (*SRY*) gene.

Statistical analysis

The normality of continuous variables was assessed using the Shapiro-Wilk's *W*-test. As the continuous variables were not normally distributed, nonparametric statistical methods were used. To compare continuous variables between two groups, the Mann-Whitney *U*-test was applied, whereas to compare them among multiple groups, the Kruskal-Wallis analysis of variance by ranks test was performed. Multiple comparisons of mean ranks for all groups were carried out as post-hoc tests. The Fisher exact and Pearson χ^2 tests were used to compare categorical variables between groups. The Spearman rank order correlation was applied to calculate correlation coefficients.

Multiple linear regression analysis and analysis of covariance (ANCOVA) were undertaken, as a non-parametric method, with logarithmically transformed values of the dependent variable. Odds ratios (OR) with 95% confidence intervals (CI) were calculated by logistic regression analyses. The diagnostic accuracy of serum α_2-HS glycoprotein measurements was evaluated using the Receiver Operating Characteristic (ROC) curve analysis.

Statistical analyses were performed using the following software: STATISTICA (version 8.0; StatSoft, Inc., Tulsa, Oklahoma, USA), Statistical Package for the Social Sciences (version 15.0 for Windows; SPSS, Inc., Chicago, Illinois, USA) and MedCalc for Windows (version 10.0.1.0; MedCalc Software, Mariakerke, Belgium). For all statistical analyses, $p<0.05$ was considered statistically significant.

Results

Circulating AHSG and CRP acute phase proteins in preeclampsia

Serum C-reactive protein levels were significantly higher, while serum α_2-HS glycoprotein concentrations were significantly lower in preeclamptic patients than in normotensive, healthy pregnant women. In the group of preeclamptic patients, no statistically significant differences were observed in serum CRP and α_2-HS glycoprotein levels between preeclamptic patients with and without fetal growth restriction. In preeclamptic patients, serum α_2-HS glycoprotein concentrations showed significant inverse correlations with systolic blood pressure and serum CRP levels. However, no other relationship was found between clinical features (maternal age, smoking status, parity, BMI and gestational age at blood draw, diastolic blood pressure, gestational age at delivery and fetal birth weight) and serum α_2-HS glycoprotein levels in preeclampsia.

Using the Receiver Operating Characteristic (ROC) curve analysis, we determined a cut-off value of AHSG concentration (720 μg/ml), which can discriminate preeclamptic patients from normotensive, healthy pregnant women with 68.1% sensitivity and 60.8% specificity. Low AHSG level (≤720 μg/ml) was significantly associated with preeclampsia (odds ratio, OR: 3.32, 95% confidence interval, CI: 1.88-5.86; $p<0.001$). We compared the diagnostic performance of serum AHSG and CRP measurements in preeclampsia and found no significant difference between the areas under the ROC curves of α_2-HS glycoprotein and CRP.

Frequency of CD4+ CD25high Foxp3+ and CD4+ CD25- Foxp3+ regulatory T cells in peripheral blood of non-pregnant, healthy pregnant and preeclamptic women

The frequency of CD4+ CD25high FoxP3+ cells was lower in non-pregnant than in healthy pregnant women and higher in healthy pregnant than in preeclamptic women. The proportion of activated CD4+ CD25high FoxP3high Tregs among CD4+ CD25high cells was also lower in non-pregnant than in healthy pregnant women and higher in healthy pregnant women than in preeclampsia.

Similarly, the frequency of CD4+ CD25- FoxP3+ cells was higher in healthy pregnant than in non-pregnant women, and lower again in preeclampsia. Within preeclamptic patients, no difference was detected in the frequency of the above subsets when patients were compared based on early or late onset of preeclampsia, severity of preeclampsia, or the presence of fetal growth restriction. Additionally, we examined the relationship between CD4+ CD25high FoxP3+ and CD4+ CD25- FoxP3+ cells in all three study groups. No difference was detected between the ratios of the above subsets in non-pregnant, healthy pregnant and preeclamptic women.

Measurement of ficolins, complement activation products, angiogenic factors and markers of endothelial activation, endothelial injury and trophoblast debris in non-pregnant, healthy pregnant and preeclamptic women

Plasma levels of ficolin-2 were significantly lower in healthy pregnant than in healthy non-pregnant women, while ficolin-3 levels did not differ significantly between the two groups. Furthermore, preeclamptic patients had significantly lower ficolin-2 and ficolin-3 concentrations than healthy non-pregnant and pregnant women. Using the Receiver Operating Characteristic (ROC) curve analysis, we determined cut-off values for plasma levels of ficolin-2 (<2.84 μg/ml; sensitivity: 70.2%, specificity: 66.1%) and ficolin-3 (<24.0 μg/ml; sensitivity: 68.3%, specificity: 54.2%) to discriminate preeclamptic patients from healthy pregnant women. Both low ficolin-2 and ficolin-3 levels were significantly associated with preeclampsia. In the group of preeclamptic patients, no statistically significant differences were found in plasma levels of ficolin-2 and ficolin-3 between patients with mild and severe preeclampsia, between patients with late and early onset of the disease, or between preeclamptic patients with and without fetal growth restriction. In healthy pregnant women, there was a statistically significant positive correlation between plasma ficolin-2 and serum PlGF concentrations, while a significant inverse correlation was observed between their ficolin-2 and sFlt-1 levels. In the preeclamptic group, plasma ficolin-2 levels showed a significant positive correlation with serum PlGF concentrations and significant inverse correlations with serum levels of sFlt-1, blood urea nitrogen and creatinine, serum lactate dehydrogenase activities, as well as with plasma VWF:Ag, fibronectin and cell-free fetal DNA concentrations.

However, after adjustment for serum sFlt-1 levels in multiple linear regression analyses, only the association between ficolin-2 and creatinine concentrations remained significant. There was no other relationship between plasma ficolin-2 or ficolin-3 levels of the study subjects and their clinical features and measured laboratory parameters – including complement activation products – in either study group.

Conclusions

1. Serum AHSG concentrations are decreased and show inverse correlations with systolic blood pressure and serum CRP levels in preeclampsia. The maternal systemic inflammatory response, which involves an acute phase reaction, might account for decreased serum AHSG levels in preeclampsia.

2. The frequency of conventional CD4+ CD25high FoxP3+ Tregs, but also that of non-conventional CD4+ CD25- FoxP3+ Tregs is lower in preeclampsia compared to healthy pregnancy, which suggests that the presence of both of these Treg subsets in circulation is similarly important in the adequate development of pregnancy-specific immune tolerance. Our data indicate that the expression of FoxP3 is a more important factor in Treg function, and a better marker of Tregs in pregnancy than CD25.

3. Circulating levels of ficolin-2 are decreased in the third trimester of normal pregnancy. There is a further decrease in plasma ficolin-2 concentrations in preeclampsia, which might contribute to the development of the maternal syndrome of the disease through impaired removal of the trophoblast-derived material released into the maternal circulation by the hypoxic and oxidatively stressed preeclamptic placenta.

List of publications

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