Serum angiogenic factor and cytokine profile in preeclampsia

Ph.D. Thesis

András Szarka, M.D.

Clinical Medicine Doctoral School
Semmelweis University

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

Official reviewers: Andrea Molnár, M.D., Ph.D.
Richárd Szmola, M.D., Ph.D.

Head of the Final Examination Committee:
Professor György Siklósi, M.D., D.Sc.
Members of the Final Examination Committee:
Ibolya Czegle, M.D., Ph.D.
Pál Siklós, M.D., C.Sc.

Budapest
2012

Introduction

Preeclampsia, characterized by hypertension and proteinuria developing after midgestation, is a severe complication of human pregnancy with a worldwide incidence of 2-8%. 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 with activation of both the innate and adaptive arms of the immune system, as well as an imbalance between circulating angiogenic factors and their antagonists plays a central role in the pathogenesis of the disease. The development of preeclampsia is influenced by both genetic and environmental risk factors, suggesting its multifactorial inheritance.

Soluble fms-like tyrosine kinase-1 (sFlt-1), the naturally occurring soluble form of vascular endothelial growth factor receptor 1 (VEGFR1), is produced by alternative splicing of the Flt-1 transcript, resulting in a deletion of the intracellular and transmembrane domains of Flt-1. sFlt-1 binds VEGF and placental growth factor (PlGF) with high affinity, acting as a soluble trap of these angiogenic factors. Placental sFlt-1 was found to be up-regulated in preeclampsia, leading to increased circulating levels of sFlt-1 that fell after delivery. Increased serum sFlt-1 levels in patients with preeclampsia were associated with decreased circulating levels of free VEGF and PlGF. Administration of sFlt-1 to pregnant rats induced hypertension, proteinuria and glomerular endotheliosis, the hallmarks of preeclampsia. Experimental data indicate that placental hypoxia is responsible for increased sFlt-1 expression in preeclamptic placenta. Furthermore, it has been demonstrated that increased circulating levels of sFlt-1 and reduced levels of free PlGF predict the subsequent development of preeclampsia.

An important feature of systemic inflammation in preeclampsia is the absence of Th2 skewness characteristic for healthy pregnancy, and thus the predominance of Th1-type immunity. Saito et al. reported firstly that the percentage of Th1 cells and the ratios of Th1/Th2 were significantly higher, while the percentage of Th2 cells was significantly lower in the peripheral blood in preeclampsia than in the third trimester of normal pregnancy. In another study, this group observed increased production of interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-α by peripheral blood mononuclear cells (PBMCs) in preeclampsia and, interestingly, positive

correlations between mean blood pressure and concentrations of Th1 cytokines. The shift to a predominant Th1-type immunity in preeclampsia was reinforced by other experiments on intracellular cytokine measurements in peripheral blood T (both helper and cytotoxic) cells and NK cells, as well as by assessment of cytokine secretion levels of PBMCs isolated from preeclamptic patients. However, the studies on circulating levels of cytokines in normal pregnancy and preeclampsia yielded conflicting results.

Heat shock proteins (Hsps) are ubiquitous and phylogenetically conserved molecules, which indicate their functional importance. They are usually considered to be intracellular proteins with molecular chaperone and cytoprotective functions. However, 70 kDa heat shock protein (Hsp70, HSPA1A) is present in the peripheral circulation of healthy non-pregnant and pregnant individuals. Serum Hsp70 levels are increased and reflect systemic inflammation, oxidative stress and hepatocellular injury in preeclampsia. Furthermore, serum Hsp70 levels are significantly higher in patients with the syndrome of hemolysis, elevated liver enzymes, and low platelet count (HELLP syndrome) than in severely preeclamptic patients without HELLP syndrome. Elevated serum Hsp70 level indicates tissue damage (hemolysis and hepatocellular injury) and disease severity in HELLP syndrome. Nevertheless, circulating levels of anti-heat shock protein antibodies (anti-human Hsp60, anti-human Hsp70, anti-mycobacterial Hsp65) are not affected in preeclampsia.

Objectives

1. There is an increasing body of evidence that an imbalance between circulating angiogenic and anti-angiogenic factors plays an important role in the pathogenesis of preeclampsia. Therefore, we determined serum sFlt-1 and PlGF levels in healthy non-pregnant and pregnant women and preeclamptic patients. We also measured several markers of processes involved in the pathogenesis of preeclampsia, and investigated whether the clinical characteristics and laboratory parameters of the study participants, including markers of inflammation (C-reactive protein), endothelial activation (von Willebrand factor antigen), endothelial injury (fibronectin), oxidative stress (malondialdehyde) and trophoblast debris (cell-free fetal DNA), were related to their serum sFlt-1 and PlGF levels. We also aimed to determine the diagnostic accuracy of serum sFlt-1 and PlGF measurements in preeclampsia.
2. Preeclampsia is characterized by an excessive maternal systemic inflammatory response with activation of both the innate and adaptive arms of the immune system. Cytokines, chemokines and adhesion molecules are central to innate and adaptive immune processes. Therefore, our aim was to determine circulating levels of cytokines, chemokines and adhesion molecules in a comprehensive manner involving a large number of healthy non-pregnant and pregnant women and preeclamptic patients. We also investigated whether serum cytokine, chemokine and adhesion molecule levels were related to the clinical characteristics and laboratory parameters of the study participants, including markers of overall inflammation (C-reactive protein), endothelial activation (von Willebrand factor antigen), endothelial injury (fibronectin), oxidative stress (malondialdehyde) and trophoblast debris (cell-free fetal DNA).
3. We have previously reported that serum Hsp70 levels are increased and reflect systemic inflammation, oxidative stress and hepatocellular injury in preeclampsia. We extended our previous observations and examined whether increased serum Hsp70 concentrations in women with preeclampsia are related to circulating levels of cytokines, chemokines, adhesion molecules and angiogenic factors, the key players in the pathogenesis of the disease.

Patients and methods

Study participants

Sixty preeclamptic patients, 60 healthy pregnant women with uncomplicated pregnancies and 59 healthy non-pregnant women were involved in our case-control study. 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. The preeclamptic patients and healthy pregnant women were matched on the basis of maternal age and gestational age at blood draw, and they were selected accordingly from 93 preeclamptic patients and 176 healthy pregnant women. Exclusion criteria were multifetal gestation, chronic hypertension, diabetes mellitus, autoimmune disease, angiopathy, renal disorder, maternal or fetal infection and fetal congenital anomaly. The healthy non-pregnant women were consecutively selected in the early follicular phase of their menstrual cycle (between cycle days 3 and 5), and none of them received hormonal contraception. The women were fasting, none of the pregnant women were in active labor, and none had rupture of membranes.

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 were not enrolled in this study. Early onset of preeclampsia was defined as onset of the disease before 34 weeks of gestation. 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 total sFlt-1 and biologically active 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) in 54 preeclamptic patients, 58 healthy pregnant women and 52 healthy non-pregnant women. Serum levels of IL-1β, IL-1 receptor antagonist (IL-1ra), IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p40, IL-12p70, IL-18, IFN-γ, TNF-α, interferon-γ-inducible protein (IP)-10, monocyte chemotactic protein (MCP)-1, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 were determined by multiplex suspension array (Bio-Plex, Cat. No. X500317TGY and XF0000ZGAI) on a Bio-Plex 200 analyzer (Bio-Rad Laboratories, Hercules, California, USA). Levels of transforming growth factor (TGF)-β1 in maternal sera were assessed by enzyme-linked immunosorbent assay (ELISA) (DRG International, Mountainside, New Jersey, USA, Cat. No. EIA-1864). Serum Hsp70 (HSPA1A) levels were measured in 60 preeclamptic patients and 60 healthy pregnant women with uncomplicated pregnancies by using the ELISA Kit of R&D Systems (DYC1663E, Minneapolis, Minnesota, USA). Standard laboratory parameters (clinical chemistry) and C-reactive protein (CRP) levels 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. 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. Plasma malondialdehyde levels were measured by the thiobarbituric acid-based colorimetric assay.

Statistical analysis

The normality of continuous variables was assessed using the Shapiro-Wilk's *W*-test. As the continuous variables were not normally distributed, non-parametric 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 (ANOVA) 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. The diagnostic accuracy of serum sFlt-1 and PlGF measurements in preeclampsia was evaluated with the Receiver Operating Characteristic (ROC) curve analysis. The scatterplots were created and multiple linear regression analyses 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.

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. Data are presented as median (interquartile range) for continuous variables and as number (percentage) for categorical variables.

Results

Circulating angiogenic factors in preeclampsia

Serum levels of sFlt-1 and PlGF, as well as sFlt-1/PlGF ratio, were significantly higher in healthy pregnant than non-pregnant women. Preeclamptic patients had significantly higher sFlt-1 levels and sFlt-1/PlGF ratio and significantly lower PlGF concentrations as compared to healthy pregnant women. Moreover, their sFlt-1 and PlGF levels and sFlt-1/PlGF ratio were significantly higher than those of healthy non-pregnant women.

According to the subgroup analyses, severely preeclamptic patients had significantly higher sFlt-1 levels compared to those with mild preeclampsia. In addition, preeclamptic patients with fetal growth restriction or onset of the disease before term (<37 weeks of gestation) had significantly lower PlGF concentrations than those without IUGR or with a disease onset at term (≥37 weeks).

In healthy non-pregnant women, we found a statistically significant negative correlation between serum PlGF concentrations and systolic blood pressure. In the group of healthy pregnant women, primiparas had significantly higher serum sFlt-1/PlGF ratio than multiparas. Serum sFlt-1 levels of healthy pregnant women showed significant positive correlations with serum creatinine levels, as well as with plasma levels of VWF:Ag and fibronectin. A significant positive correlation was observed between PlGF levels of healthy pregnant women and fetal birth weight, while their PlGF and CRP concentrations correlated inversely with each other. In the preeclamptic group, there were significant positive correlations between serum sFlt-1 levels and systolic and diastolic blood pressure, serum levels of blood urea nitrogen (BUN) and creatinine, as well as plasma levels of VWF:Ag, fibronectin and cell-free fetal DNA. Furthermore, serum PlGF concentrations of preeclamptic patients showed significant positive correlations with gestational age at disease onset and delivery, as well as with fetal birth weight, and significant inverse correlations with serum levels of BUN, creatinine and plasma levels of fibronectin.

Using the Receiver Operating Characteristic (ROC) curve analysis, we determined cut-off values for sFlt-1 and PlGF concentrations and their ratio to

discriminate preeclamptic patients from healthy pregnant women. The sensitivities and specificities of these cut-off points were as follows: for high sFlt-1 level (>4165 pg/ml): 74.1% and 67.2%, for low PlGF level (<146 pg/ml): 77.8% and 70.7%, and for high sFlt-1/PlGF ratio (>31.2): 75.9% and 74.1%, respectively.

We also compared the diagnostic performance of serum sFlt-1 and PlGF concentrations and their ratio in preeclampsia. The area under the ROC curve (AUC) of sFlt-1/PlGF ratio was significantly higher than that of sFlt-1 and PlGF levels (AUC with 95% confidence interval for sFlt-1/PlGF ratio, sFlt1 and PlGF levels were 0.81 (0.73-0.88) versus 0.75 (0.66-0.83) and 0.77 (0.68-0.85), respectively; $p<0.05$ for both).

Serum cytokine profile in normal pregnancy and preeclampsia

Apart from serum IL-1β and TGF-β1 levels, circulating levels of cytokines, chemokines and adhesion molecules differed significantly among our study groups. There were no significant differences in the ratios of IL-2 to IL-4 and IFN-γ to IL-4 between healthy non-pregnant and pregnant women, whereas these ratios were significantly increased in preeclamptic patients as compared to healthy pregnant women. On the contrary, IL-18/IL-12p70 ratios were significantly higher, while IL-12p70/IL-12p40 ratios were significantly lower in healthy pregnant than in non-pregnant women, but they showed the same level in preeclamptic patients compared with healthy pregnant women. In addition to a shift towards Th1-type immunity (expressed by the increased IL-2/IL-4 and IFN-γ/IL-4 ratios), circulating levels of the pro-inflammatory cytokines IL-6 and TNF-α, the chemokines IL-8, IP-10 and MCP-1, as well as the adhesion molecules ICAM-1 and VCAM-1, were raised in preeclampsia compared with healthy pregnancy, resulting in an overall pro-inflammatory systemic environment.

In the group of preeclamptic patients, no statistically significant differences were found in serum levels of the measured cytokines, chemokines and adhesion molecules 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 non-pregnant women, serum IL-6 and TNF-α concentrations correlated significantly with CRP levels. In the group of healthy pregnant women, we found statistically significant negative correlations between serum IL-2 and IFN-γ concentrations and gestational age at delivery. A significant positive correlation was observed between IL-6 and CRP levels of healthy pregnant women, while their TGF-β1 and malondialdehyde concentrations correlated inversely with each other. Serum IP-10 levels of healthy pregnant women showed significant positive correlations with serum creatinine levels, as well as with plasma levels of VWF:Ag and fibronectin, while a significant inverse correlation with fetal birth weight. Furthermore, there were significant positive correlations between their serum MCP-1 concentrations and serum creatinine, as well as plasma fibronectin levels. Serum IP-10 levels of preeclamptic patients correlated significantly with serum BUN and creatinine levels, serum aspartate-aminotransferase (AST), alanine-aminotransferase (ALT) and lactate-dehydrogenase (LDH) activities, as well as with plasma VWF:Ag and fibronectin concentrations. In the preeclamptic group, we found significant positive correlations between serum MCP-1 levels and systolic blood pressure, serum CRP, as well as plasma malondialdehyde concentrations. ICAM-1 concentrations of preeclamptic patients showed significant positive correlations with bilirubin, CRP and malondialdehyde levels, as well as with AST and LDH activities, while their VCAM-1 concentrations correlated with BUN, creatinine and fibronectin levels, as well as with AST and LDH activities.

Relationship of serum 70 kDa heat shock protein (Hsp70, HSPA1A) levels to circulating cytokines, chemokines, adhesion molecules and angiogenic factors in women with preeclampsia

Serum levels of Hsp70 were significantly higher in preeclamptic patients than in healthy pregnant women. In the preeclamptic group, serum Hsp70 concentrations showed significant correlations with serum levels of IL-12p40, MCP-1, ICAM-1 and VCAM-1. The associations remained significant even after adjustment for age and gestational age at blood draw in multiple linear regression analyses.

Using the ROC curve analysis, we determined cut-off values for serum Hsp70 concentration (>0.34 ng/ml; sensitivity: 86.7%, specificity: 86.7%) and sFlt-1/PlGF

ratio (>31.2; sensitivity: 75.9%, specificity: 74.1%) to discriminate preeclamptic patients from healthy pregnant women. Women with elevated serum Hsp70 level and sFlt-1/PlGF ratio had substantially higher risk for preeclampsia than those with elevated serum Hsp70 concentration or sFlt-1/PlGF ratio alone (OR (95% CI): 72.4 (17.4-300), $p<0.001$ versus 8.14 (1.82-36.5), $p<0.05$ and 0.42 (0.05-3.72), $p>0.05$, respectively), even after adjustment for age and gestational age at blood collection in multiple logistic regression analyses (adjusted OR (95% CI): 82.6 (17.9-381), $p<0.001$ versus 7.80 (1.53-39.7), $p<0.05$ and 0.38 (0.04-3.44), $p>0.05$, respectively).

Conclusions

1. Elevated serum sFlt-1 and decreased serum PlGF concentrations were found to be associated with systolic and diastolic blood pressure, renal and endothelial dysfunction, trophoblast deportation process, as well as with a shorter duration of pregnancy, fetal growth restriction, the severity and preterm onset of the disease in preeclampsia. However, maternal age, BMI, smoking status, parity, gestational age at blood collection, liver function parameters, as well as markers of inflammation and oxidative stress were not related to circulating levels of angiogenic factors in our preeclamptic patients. These findings indicate the central role of an angiogenic imbalance in the pathogenesis of this pregnancy-specific disorder.
2. In our study, we determined circulating levels of several cytokines, chemokines and adhesion molecules in healthy non-pregnant and pregnant women and preeclamptic patients by high-throughput multiplex suspension array technology. Except for serum IL-1β and TGF-β1 levels, all of the measured inflammatory variables differed significantly among the three study groups. According to our findings, preeclampsia is associated with an overall pro-inflammatory systemic environment. Elevated amounts 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 characteristics of the maternal syndrome of preeclampsia.
3. According to our results, increased serum Hsp70 concentrations in women with preeclampsia are associated with pro-inflammatory changes in circulating cytokine profile, suggesting that circulating Hsp70 might contribute to the development of the generalized intravascular inflammatory reaction characteristic of the maternal syndrome of the disease. Furthermore, the combination of elevated serum Hsp70 level and sFlt-1/PlGF ratio was found to be additive for the risk of preeclampsia.

List of publications

Publications related to the subject of the thesis

1. Molvarec A, **Szarka A**, Walentin S, Szűcs E, Nagy B, Rigó J Jr. (2010) Circulating angiogenic factors determined by electrochemiluminescence immunoassay in relation to the clinical features and laboratory parameters in women with pre-eclampsia. Hypertens Res, 33: 892-898. (IF: 2.353)
2. **Szarka A**, Rigó J Jr, Lázár L, Bekő G, Molvarec A. (2010) Circulating cytokines, chemokines and adhesion molecules in normal pregnancy and preeclampsia determined by multiplex suspension array. BMC Immunol, 11: 59. (IF: 3.0)
3. Molvarec A, **Szarka A**, Walentin S, Bekő G, Karádi I, Prohászka Z, Rigó J Jr. (2011) Serum heat shock protein 70 levels in relation to circulating cytokines, chemokines, adhesion molecules and angiogenic factors in women with preeclampsia. Clin Chim Acta, 412: 1957-1962. (IF: 2.535)

Publications independent of the subject of the thesis

1. **Szarka A**, Gerlei Zs, Berkes E, Kóbori L, Molvarec A, Garamvölgyi Z, Rigó J Jr. (2008) Májtranszplantációt követően sikeresen kiviselt terhesség [A case of a successful pregnancy and delivery after liver transplantation]. Magy Nőorv L, 71(6): 277-279.
2. Molvarec A, Kalabay L, Derzsy Z, **Szarka A**, Halmos A, Stenczer B, Arnaud P, Karádi I, Prohászka Z, Rigó J Jr. (2009) Preeclampsia is associated with decreased serum alpha(2)-HS glycoprotein (fetuin-A) concentration. Hypertens Res, 32: 665-669. (IF: 2.426)
3. Molvarec A, Shiozaki A, Ito M, Toldi G, Stenczer B, **Szarka A**, Nakashima A, Vásárhelyi B, Rigó J Jr, Saito S. (2011) Increased prevalence of peripheral blood granulysin-producing cytotoxic T lymphocytes in preeclampsia. J Reprod Immunol, 91: 56-63. (IF: 2.966)
4. Molvarec A, **Szarka A**, Walentin S, Bekő G, Karádi I, Prohászka Z, Rigó J Jr. (2011) Serum leptin levels in relation to circulating cytokines, chemokines,

5. Stenczer B, Molvarec A, Szabó G, **Szarka A**, Fügedi G, Szijártó J, Rigó J Jr. (2012) Circulating levels of thrombospondin-1 are decreased in HELLP syndrome. Thromb Res, 129: 470-473. (IF: 2.440)
6. Lázár L, Nagy B, Molvarec A, **Szarka A**, Rigó J Jr. (2012) Role of hsa-miR-325 in the etiopathology of preeclampsia. Mol Med Report, 6: 597-600. (IF: 0.418)
7. Wettstein D, Gerlei Zs, Rigó J Jr, **Szarka A**, Asztalos L, Langer R. (2012) Szolid szerv transzplantációját követő gyermekvállalás Magyarországon [Childbirth after solid organ transplantation in Hungary]. Magy Nőorv L, 75(4): 4-7.

Cumulative impact factor: 18.183.