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Angiogenic factor screening in women with mild preeclampsia – New and significant proteins in plasma

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ABSTRACT

Introduction: The aim of this study was to analyse a panel of 60 angiogenic factors (pro-angiogenic and anti-angiogenic) in the plasma of women with mild preeclampsia.

Materials and Methods: We recruited 21 women between 25 and 40 weeks gestation with diagnosed mild pre-eclampsia into the study group and 27 healthy women with uncomplicated pregnancies of corresponding gestational age to that of the study to the control group. We used a quantitative protein macroarray method that allowed for analysis of 60 angiogenic proteins per sample simultaneously.

Results: We showed a statistically significant increase in the concentration of 8 proteins, interferon gamma (IFN-γ), interleukin 6 (IL-6), leukaemia inhibitory factor (LIF), heparin-binding EGF-like growth factor (HB-EGF), hepatocyte growth factor (HGF), C-X-C motif chemokine 10 (IP-10), leptin and platelet-derived growth factor BB (PDGF-BB), as well as a significant decrease in the concentration of 3 proteins, vascular endothelial growth factor (VEGF), placental growth factor (PlGF) and follistatin, in the plasma of women with preeclampsia.

Conclusion: Based on our findings, it seems that protein factors may play an important role in the pathogenesis of preeclampsia, and there are many proteins that have not been studied in PE to date. There are no previous studies assessing the LIF, follistatin, HGF, HB-EGF and PDGF-BB concentrations in the plasma of women with PE; therefore, our obtained results indicate that these proteins are new factors that can play an important role in the pathomechanisms of PE.

1. Introduction

Preeclampsia (PE) is a disorder that occurs in 3–5% of pregnancies in Western Europe and North America with almost 8.5 million cases per year recorded worldwide [1]. It is the most common cause of mortality in pregnant women. Clinically, mild preeclampsia (this type of PE will be studied in our project) is the first onset of this disease and is associated with hypertension $\geq$140/90 mmHg and proteinuria $\geq 0.3 < 5$ g/24 h occurring after the 20th gestational week in women previously identified as normotensive with no protein in urine [2]. It is estimated that preeclampsia will develop in almost 35% of women with gestational hypertension diagnosed before the 34th week of pregnancy. The course of preeclampsia is individually specific - it may present with varying degrees of severity of hypertension and proteinuria and may be complicated by HELLP syndrome (haemolytic anaemia, elevated liver enzymes, low platelet count) as well as severe eclampsia [3]. The associated symptoms are generalized edema, headache and blurred vision, and in severe cases, it may cause liver failure, kidney disease, coagulation disorders, respiratory distress syndrome and intrauterine foetal growth restriction [2]. Despite many hypotheses, the pathogenesis of pre-eclampsia has not been clearly established, and the most effective 'remedy' is delivery [4]. There are many risk factors for

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preeclampsia, including obesity, abnormal lipid profile, type 2 diabetes, insulin resistance, gestational diabetes, maternal age below 20 years of age and above 35 years of age, multiple pregnancy, first pregnancy, urinary tract infection, family history of preeclampsia and cardiovascular disease occurring before pregnancy [5].

For normal growth and development of the foetus, it is essential to reconstruct the spiral arteries in the uterine wall between 10 and 16 weeks of gestation. The process is divided into three main stages according to structural criteria: (1) vascular changes independent of cytotrophoblast invasion; (2) vascular remodelling induced by perivascularly located cytotrophoblasts; and (3) cytotrophoblast infiltration into the spiral arteries [6,7]. As a result of these transformations, spiral arteries change from arteries with low blood flow and high resistance into arteries with fast blood flow and low resistance, ensuring an adequate flow of blood through the placenta and thus normal development of the foetus [4]. In preeclampsia, invasion of the vessel walls by trophoblasts is impaired, which causes the spiral arteries to narrow and results in insufficient delivery of oxygen and nutrients to the foetus for its proper development [4]. Placental ischemia triggers the release of placenta lipids and protein factors and upsets the balance of angiogenic factors, which induces endothelial dysfunction [8,9]. Therefore, measurement of angiogenic factors in women with preeclampsia could lead to a better understanding of the pathomechanisms of this disease and possibly provide new information about the processes that occur in the body of a pregnant woman with PE. The aim of this study was to profile the major angiogenesis-related factors in the plasma of patients with mild preeclampsia. The 60 proteins analysed by our team are the most important factors in human circulation and play a major role in the pro and anti-angiogenic pathways.

2. Material and methods

The recruitment of patients to the study and control groups started after 24 weeks of gestation since we attempted to have all patients undergo a 75 g Oral Glucose Tolerance Test (which is routinely conducted in Poland between the 24th and 28th week in pregnant women). We recruited patients with mild preeclampsia between 25 and 40 weeks gestation (patients with severe PE were not included because we aimed for greater homogeneity in the study group). The inclusion criteria were as follows: blood pressure between 140/90 and 160/110 mmHg in two independent measurements taken at an interval of at least 6 h, the presence of protein in the 24-h urine collection at a level above 300 mg/24 h but not more than 5 g/24 h. We excluded women with: chronic hypertension, multiple pregnancies, pre-existing diabetes or gestational diabetes, connective tissue disease, kidney disease, viral diseases, toxoplasmosis, urinary tract infection, thrombocytopenia and coagulation disorders, pregnancy diagnosed with chromosomal aberrations before or after childbirth and BMI $>$ 30 at the time of recruitment. We obtained 20 ml of blood in EDTA tubes from each patient (fasting) who qualified for the study. In accordance with the blood fractionation procedures, we obtained plasma for protein determination. Blood taken from the patient qualified for the study group or control group was prepared for 30 minutes in an ice bath. Plasma samples were kept at −80 °C until further analysis.

Our department (Perinatology and Obstetrics of the Medical University of Bialystok) eventually recruited 21 pregnant women with mild preeclampsia (study group) and 27 pregnant women (matched for maternal age, gestational age and BMI at the moment of recruitment) with uncomplicated pregnancies (control group). The study protocol was approved by the Local Ethical Committee of the Medical University of Bialystok, Poland, and informed consent was obtained from each patient (No ethics committee approval: R-I-002/529/2013). Signed informed consent from all participants involved in the study was obtained.

To assess the concentration of angiogenic factors in blood plasma, we used a multiplex method [10–12] that allows for the simultaneous determination of 60 proteins per sample. Similar to a traditional sandwich-based ELISA, the multiplex method uses a pair of specific protein antibodies for detection. A capture antibody is first bound to the glass surface, and after incubation with the sample, the target angiogenic factor is trapped on the solid surface. A second biotin-labelled detection antibody is then added that recognizes a different isotope of the target factor. The protein factor-antibody-biotin complex is then visualized by the addition of a streptavidin-labelled Cy3 equivalent dye using a laser scanner (GenePix 4100A) and two types of software: GenePix Pro7 and Q-Analyzer. Normalization of the fluorescent signal on protein arrays was performed by the software, which consisted of subtracting the background signal so that the array of spots were recorded real signals derived from proteins.

To validate of our results from macroarrays, we used ELISA (R & D System kits) for 4 statistically significant proteins: leptin, IL-6, VEGF and HGF. ELISA is a reference method, which quantitatively measures protein concentration in biological material.

The sets (Human Angiogenesis Array 1000, RayBiotech, Inc.) consist of the following angiogenic factors: Activin A, Agouti-related protein (AgRP); Angiopoietin 1; Angiopoietin 2; Angiogenin; Angiostatin; Angiopoietin-like 4 (ANGPTL4); Basic fibroblast growth factor (bFGF); Chemokine (C-X-C motif) ligand 16 (CXCL16); Epidermal growth factor (EGF); C-X-C motif chemokine 5 (ENA-78); Fibroblast growth factor 4 (FGF-4); Follistatin; Granulocyte-colony stimulating factor (G-CSF); Granulocyte-macrophage colony-stimulating factor (GM-CSF); Chemokine (C-X-C motif) ligand 1 (GRO); Heparin-binding EGF-like growth factor (HB-EGF); Hepatocyte growth factor (HGF); Chemokine (C-C motif) ligand 1 (I-309); Interferon gamma (IFN-gamma); Insulin-like growth factor 1 (IGF-1); Interleukin 10 (IL-10); Interleukin 12 p40 (IL-12 p40); Interleukin 12 p70 (IL-12 p70); Interleukin 17 (IL-17); Interleukin 1-alpha (IL-1 alpha); Interleukin 1-beta (IL-1 beta); Interleukin 2 (IL-2); Interleukin 4 (IL-4); Interleukin 6 (IL-6); Interleukin 8 (IL-8); C-X-C motif chemokine 10 (IP-10); Chemokine (C-X-C motif) ligand 11 (I-TAC); Leptin; Leukaemia inhibitory factor (LIF); Monocyte chemotactic protein 1 (MCP-1); Monocyte chemotactic protein 2 (MCP-2); Monocyte chemotactic protein 3 (MCP-3); Monocyte chemotactic protein 4 (MCP-4); Matrix metalloproteinase-1 (MMP-1); Matrix metalloproteinase-9 (MMP-9); Platelet-derived growth factor BB (PDGF-BB); Platelet endothelial cell adhesion molecule (PECAM-1); Placental growth factor (PLGF); Chemokine (C-C motif) ligand 5 (RANTES); Transforming growth factor alpha (TGF alpha); Transforming growth factor beta 1 (TGF beta 1); Transforming growth factor beta 3 (TGF beta 3); Tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (Tie-1); Tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2); Metallopeptidase inhibitor 1 (TIMP-1); Metallopeptidase inhibitor 2 (TIMP-2); Tumour necrosis factor alpha (TNF alpha); Tumour necrosis factor beta (TNF beta); Thrombopoietin (TPO); Urokinase receptor (uPAR); Vascular endothelial growth factor (VEGF); Vascular endothelial growth factor 2 (VEGFR2); Vascular endothelial growth factor 3 (VEGFR3); and Vascular endothelial growth factor D (VEGF-D).

Descriptive statistics, including the mean concentration and standard error of the mean concentration, were calculated for selected angiogenic factors, henceforth called features. To detect statistically significant differences between the groups under consideration (study group versus control group), either an analysis of variance model [13] was fit or a nonparametric method (Wilcoxon rank-sum test [14]) was applied. The choice of an appropriate method was made upon fulfilling the normality and homogeneity of variances assumptions; in the case of a violation of at least one condition, a nonparametric approach was employed.

The normality of the distribution of features was checked with the Shapiro-Wilk test [15] and the homogeneity of variances with Levene's test [16]. Features that were found to be significant, that is, their distribution was statistically significantly different among the experimental groups, were taken under further investigation to determine