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THE PATHOPHYSIOLOGY OF ENDOTHELIAL FUNCTION IN PREGNANCY AND THE USEFULNESS OF ENDOTHELIAL MARKERS

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Aim. The aim of this study was to assess coagulation markers of endothelial damage and examine new markers of endothelial activation such as matrix metalloproteinases (MMPs) in a group of healthy pregnant women. Matrix metalloproteinase (MMP)-2, in particular, plays a major role in the degradation of the extracellular matrix confirming its essential function in both the survival (angiogenesis) and death of endothelial cells.

Detection of specific coagulation factors, mainly released from the vascular endothelium such as vWF, sTM (soluble thrombomodulin) and ePCR (endothelial protein C receptor) and factors dependent on endothelial activation such as t-PA and PAI-1, could provide information on possible endothelial dysfunction and help differentiate pregnant patients with an altered thrombotic state.

Methods. Healthy pregnant women underwent complete assessment for endothelial damage (as vWF, vWF activity, sTM, ePCR, EMP, MMP-2, MMP-9 and TIMP-2) using the ELISA and other methods.

Results and Conclusions. The results show that endothelial activation during pregnancy is different from that in other pathological conditions involving endothelial damage and typically characterized by higher levels of both coagulation endothelial markers and MMPs. In pregnancy, changes in extracellular matrix composition and matrix metalloproteinase activity also occur and promote vascular remodeling but, only in the uterus. Predisposing risk factors for epithelial dysfunction, and vascular mediators associated with vascular remodeling must be assessed from concentrations in whole blood. The levels of MMPs are not increased in the circulation and the local situation in the uterus cannot be monitored this way.

However, MMP-2 processes and modulates the functions of many other vasoactive and pro-inflammatory molecules including adrenomedullin, big endothelin-1, calcitonin gene-related peptide, CCL7/MCP-3, CXCL12/SDF-1, galectin-3, IGFBP-3, IL-1 Beta, S100A8, and S100A9. These molecules represent new potential molecular markers of endothelial damage during pregnancy.

INTRODUCTION

The healthy endothelium secretes and expresses a number of substances that help to maintain vascular wall structure and vascular homeostasis. Endothelial cells modulate their properties to restore vascular homeostasis in response to various aggressive stimuli[1]. Under some pathological conditions however, such as atherosclerosis, endothelial function is chronically disturbed. Advances in recent years have improved our understanding of the pathophysiology of atherosclerosis which is now known to be a dynamic and progressive process proceeding from endothelial dysfunction and inflammation of the vascular wall. The progression of the atherosclerosis and its prognosis, along with the efficacy of therapeutic modalities, can be assessed by measuring the circulating levels of various biomarkers expressed/released by the endothelium. The endothelium in pregnancy on the other hand is less well understood[2]. The purpose of this paper is to reconsider the importance of markers of endothelial function under physiological conditions in normal and pathological pregnancies, and to analyze markers of endothelial function with clinical applicability.

Hypertension and preeclampsia in pregnancy are multisystemic conditions characterized by hypertension, proteinuria and generalized systemic vasoconstriction. Preeclampsia is associated with insufficient trophoblast invasion into maternal spiral arteries. These changes lead to insufficient fetoplacental blood flow. Ischemia of the fetoplacental unit causes the release of specific factors into maternal vessels and subsequent activation of the endothelium and, vasoconstriction. While these factors have not been completely identified so far, it has been found that in the manifest phase of preeclampsia, there is a significantly higher level of markers connected with endothelial damage.

In this study, we compared coagulation markers of endothelial damage and new markers of endothelial activation such as matrix metalloproteinases. Matrix metalloproteinase (MMP)-2 in particular, plays a major role in the degradation of the extracellular matrix and this underlies the essential function of this enzyme in both the survival (angiogenesis) and death of endothelial cells. Endothelial cells however cannot be detected in blood samples, owing to their very low concentration. To detect the activation mechanism of MMP, analysis of endothelial microparticles (EMPs) is used. The precise mechanisms leading to *in vivo* microparticle generation by endothelial cells remain unclear. *In vitro*, a variety of prolonged stimuli have been shown to induce EMP vesiculation from cultured endothelial cells. Combes et al.[3] first described the generation of EMPs from human umbilical endothelial cells stimulated by tumor necrosis factor-α. However, EMP release can be triggered by non-apoptotic stimuli as well[4–7].

The detection of specific coagulation factors, mainly released from the vascular endothelium such as vWF, sTM and ePCR and factors dependent on endothelial activation such as t-PA and PAI-1, could provide information on possible endothelial dysfunction and help differentiate pregnant patients with an altered thrombotic state. The Von Willebrand factor is a high molecular weight glycoprotein and has been proposed as a marker of endothelial dysfunction for many years. It is mainly responsible for platelet adhesion in the subendothelial space and for the initiation of thrombotic events. Thrombomodulin is also a high molecular glycoprotein produced by the endothelial cells. Its role in hemostasis is crucial. It is expressed through the activation and transformation of thrombin from prethrombotic to antithrombotic agent. The endothelial protein C receptor (EPCR) functions as an important regulator of the protein C anticoagulant pathway by binding protein C and enhancing activation through the thrombin-thrombomodulin complex. EPCR binds to both protein C and activated protein C (APC) with high affinity. A soluble form of EPCR (sEPCR) circulates in plasma and inhibits APC anticoagulant activity especially during pregnancy[8]. Activation of plasminogen by tissue-type plasminogen activator (t-PA) is enhanced in the presence of fibrin or on the endothelial cell surface. For monitoring these processes, the levels of t-PA and their inhibitor PAI-1 are crucial[9,10].

MATERIAL AND METHODS

Healthy pregnant women underwent a complete assessment of endothelial damage. For this purpose, blood was collected into 0.129 M sodium citrate tubes (Vacuette, Greiner) and platelet-poor plasma was prepared within two hours by centrifugation (twice at 3000 x *g* for 10 min). Whole blood and plasma fractions were stored at -80 °C until use. All samples were tested by the same laboratory, and one aliquot of each plasma sample was thawed at 37 °C immediately before the assay. For EMP measurement, 5 ml of blood were collected by venipuncture into 0.129 M trisodium citrate. Platelet-free plasma was prepared within one hour by two sequential centrifugations at 1 000 x *g* for 10 min.

t-PA and PAI-1

Tissue-type plasminogen activator antigen and plasminogen activator inhibitor-1 levels were determined by the ELISA method (Technoclone GmbH, Austria).

Von Willebrand factor

Von Willebrand factor antigen levels were determined by enzyme immunoassay (Instrumentation Laboratory, Italy).

Soluble thrombomodulin antigen and endothelial protein C receptor levels were determined by the ELISA method (Diagnostica Stago, France).

Endothelial microparticles

EMPs were quantified in plasma as previously described[11]. Thirty µl of platelet-free plasma were incubated for 30 min at room temperature, with 10 µl of PE-conjugated CD144. The samples were then diluted in 1.0 ml PBS and a known number of fluorescent latex beads (Flowcount, Beckman Coulter Immunotech, France) were added to samples according to the internal standards before flow cytometry analysis. The EMPs were analyzed using Coulter Epics XL (Beckman Coulter, Switzerland, Nyon) as previously described[11]. Using 0.8-µm latex beads, EMP were defined as elements less than 1 µm in size and positively labeled with PE-conjugated CD144. The results were expressed as the number of EMPs per 1 ml of plasma.

Matrix metalloproteinases

The MMP-2 and MMP-9 activity in plasma samples was measured by the solid-phase ELISA method[12] with EDANS/DABCYL FRET peptide (AnaSpec, USA). The results were expressed in reference fluorescence units (RFU). The RFU is a unit of measurement used in ELISA methods employing fluorescence detection. Fluorescence is detected as labeled fragments, conjugated on solid phase, and excited by laser, past a detector. The software interprets the results, calculating the quantity of the fragments from the fluorescence intensity for each sample.

Tissue inhibitor of metalloproreinase-2

The TIMP-2 activity in plasma samples was measured by solid-phase ELISA (R&D Systems, USA).

Statistical analysis

The statistical analysis and graphical presentation were performed using the statistics software (StatSoft CR s. r.o. (2007). STATISTICA Cz). Clinical and laboratory data are reported as means and standard deviation (SD). Differences between trimester were analyzed using the non-parametric Wilcoxon signed-rank test and the non parametric Friedman ANOVA. Other data were analyzed using the Mann-Whitney and Krusal-Walls tests. The level of significance was set at 5%.

RESULTS

The levels of vWF antigen continued to increase throughout pregnancy (the mean levels were 1523,2 IU/l, 1733,4 IU/l and 2162,0 IU/l in the 1st, 2nd and 3rd trimesters, respectively). In the group, a statistically significant difference in levels was found between the 2nd and 3rd trimesters, with the difference between the 1st and 2nd trimesters being on the borderline of statistical significance. At the same time, vWF activity was on the rise (the average levels were 1302 IU/l, 1500,9 IU/l and 1819,1 IU/l in the 1st, 2nd and 3rd trimesters, respectively). A statistically significant difference in levels was found between the 2nd and 3rd trimesters but not between the 1st and 2nd trimesters.

The levels of thrombomodulin increased significantly during pregnancy (the average levels were 19.05 μg/l, 28.47 μg/l and 39.86 μg/l in the 1st, 2nd and 3rd trimesters, respectively). Statistically significant differences in levels were found both between the 2nd and 3rd trimesters and between the 1st and 2nd trimesters.

The levels of soluble EPCR continued to rise throughout pregnancy (the average levels were 201.76 μg/l, 274.68 μg/l and 324.07 μg/l in the 1st, 2nd and 3rd trimesters, respectively). A statistically significant difference in levels was found between the 1st and 2nd trimesters but not between the 2nd and 3rd trimesters.

The PAI-1 levels increased during the entire pregnancy (the average levels were 36.14 μg/l, 50.07 μg/l and 60.12 μg/l in the 1st, 2nd and 3rd trimesters, respectively). Statistically significant differences in levels were found both between the 2nd and 3rd trimesters and between the 1st and 2nd trimesters.

The levels of t-PA did not significantly change in the course of pregnancy (the average levels were 2.48 μg/l, 2.97 μg/l and 3.34 μg/l in the 1st, 2nd and 3rd trimesters, respectively). Similarly, no statistically significant differences were found in the levels of MMP-2 (the average levels were 9043.76 RFU [relative fluorescence units], 9315.38 RFU and 8800.27 RFU in the 1st, 2nd and 3rd trimesters, respectively), MMP-9 (the average levels were 8371.90 RFU, 8290.81 RFU and 7470.50 RFU in the 1st, 2nd and 3rd trimesters, respectively), TIMP-2 (the average levels were 92.5 μg/l, 98.5 μg/l and 96.5 μg/l in the 1st, 2nd and 3rd trimesters, respectively) and EMPs (the average counts were 3.83 $x10^9$ particles/l, 3.83$x10^9$ particles/l and 3.63 $x10^9$ particles/l in the 1st, 2nd and 3rd trimesters, respectively).

DISCUSSION

The results show that endothelial activation during pregnancy is different from that in other pathological conditions involving endothelial damage such as acute coronary syndrome or diabetes mellitus[13–17]. These pathological situations are typically characterized by higher levels of both coagulation endothelial markers and MMPs. In these conditions, the mechanism of endothelial damage is probably as follows: enhanced matrix breakdown, attributed primarily to a family of matrix metalloproteinases (MMPs) that are expressed in atherosclerotic plaques by inflammatory cells (macrophages, foam cells) and, to a lesser extent, by SMCs and endothelial cells[18]. This family of enzymes can degrade all components of the extracellular matrix[19–24].

The activity of MMPs is tightly regulated at the level of gene transcription and is also by their secretion in an inactive zymogen form that requires extracellular activation[25–27] and co-secretion of the tissue inhibitors of metalloproteinases (TIMPs) (ref.[28]).

Latent MMPs can be activated by plasmin (produced by the plasminogen activator [uPA] from plasminogen by macrophages), trypsin and chymase (derived from de-

Table 1. Comparison of the levels markers of endothelial activation by trimesters.

Markers of endothelial activation	I. trimester average level	II. trimester average level	III. trimester average level	P (the comparison I. vs II. trimester)	P (tThe comparison II. vs III. trimester)
vWf antigen	1523.2	1733.4	2162.0	0.048	0.000
vWf activity	1302.0	1500.9	1819.1	0.074	0.001
Thrombomodulin	19.05	28.47	39.86	0.000	0.000
ePCR	201.76	274.68	324.07	0.017	0.660
PAI – 1	36.14	50.07	60.12	0.000	0.012
EMP	3.83 $x10^9$	3.83 $x10^9$	3.63 $x10^9$	0.412	1.000
MMP – 2	9043.76	9315.38	8800.27	1.000	0.972
MMP – 9	8371.90	8290.81	7470.50	1.000	0.084
TIMP – 2	1.85	1.97	1.93	0.506	1.000
t-PA	2.48	2.97	3.34	0.85	0.96

granulating mast cells[29–33]). Increased MMP production can be induced by oxidized lipids, reactive oxygen species, CD-40 ligation, inflammatory cytokines, tenascin-C derived from macrophages, and hemodynamic stress[34–38].

The situation during pregnancy is different[39]. Changes in extracellular matrix composition and matrix metalloproteinase activity also promote vascular remodeling but only in the uterus. Assessment of the predisposing risk factors, the biologically active factors, and the vascular mediators associated with vascular remodeling must be made from the concentration of risk factors in the whole blood circulation, regulation mechanisms of molecules and other interactions.

From this viewpoint, it is not possible to fully clarify the pathophysiology of endothelial dysfunction during pregnancy. The levels of MMPs are not increased in the circulation and the local situation in the uterus cannot be monitored this way.

While the amounts of newly synthesized MMPs are regulated mainly at the levels of transcription, the proteolytic activities of existing MMPs are controlled through the activation of proenzymes and the inhibition of active enzymes by endogenous inhibitors such as β2-macroglobulin and TIMPs locally in the uterus[40]. TIMP-1 through TIMP-4 inhibit active MMP-2 through tight but non-covalent binding of their N-terminal domains to the catalytic domain of MMP-2 in a 1:1 stoichiometry[41–43]. In addition, TIMP-2 and TIMP-3 can tether pro-MMP-2 into cell surface ternary complexes with MT-MMPs .

Together with MMP-9 (gelatinase B), MMP-2 degrades gelatin (denatured collagen) and type IV collagen, the major component of basement membranes[44]. It can also degrade the following matrix proteins: collagens V, VII, and X, decorin, elastin and fibronectin[45–47].

MMP-2 processes and modulates the functions of many other vasoactive and pro-inflammatory molecules including adrenomedullin, big endothelin-1, calcitonin gene-related peptide, CCL7/MCP-3, CXCL12/SDF-1, galectin-3, IGFBP-3, IL-1 β, S100A8, and S100A9. These molecules represent new potential molecular markers of endothelial damage during pregnancy.

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