Placenta 32 (2011) 63–69

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

# Protein composition of microparticles shed from human placenta during placental perfusion: Potential role in angiogenesis and fibrinolysis in preeclampsia☆

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ARTICLE INFO

*Article history:*
Accepted 22 October 2010

*Keywords:*
Placental microparticles
Dual perfusion
Preeclampsia
Flt-1
Endoglin
Plasminogen activators

ABSTRACT

Shedding of syncytiotrophoblast microparticles (MPs) from placenta to maternal blood occurs in normal pregnancy and is enhanced during preeclampsia (PE). The syncytiotrophoblast synthesizes plasminogen activator inhibitors (PAIs) which regulate fibrinolysis, as well as soluble forms of the fms-like tyrosine kinase (sFlt-1) and endoglin, which exert anti-angiogenic actions. An increase in the ratio of PAI-1/PAI-2 and elevated levels of sFlt-1 and sEng in maternal serum are linked to placental damage and maternal endothelial cell dysfunction in PE. The goal of the current study was to determine whether MPs released to maternal perfusate during dual perfusion contain these factors associated with placental pathophysiology in PE. Initially, high levels of alkaline phosphatase activity and Annexin V binding were found in MPs isolated by sequential centrifugation of maternal perfusates at 10,000 and 150,000×g(10 K and 150 K MPs), indicating their plasma membrane origin. ELISA revealed the presence of these factors at the following relative levels: Eng>PAI-2⋙PAI-1>sFlt-1. Based on comparisons of their concentration in perfusates, MPs, and MP-free 150 K supernatants, we determined that MPs constitute a significant portion of Eng released by placenta. Flow cytometric analysis of 10 K MPs supported the levels of expression found by ELISA and indicated that Eng and PAI-2 were almost exclusively localized to the surface of MPs, a site with biological potential. These results indicate that MPs shed from the syncytial surface express factors which may alter the fibrinolytic and angiogenic balance at the maternal–fetal interface and play a role in the pathophysiology of PE.



## 1. Introduction

Placental microparticles (MPs) are vesicles shed from the outer layer of the placenta (i.e. the syncytium) directly to maternal blood [1–4]. The process of placental release of MPs is suggested to occur as a result of apoptosis and membrane blebbing [5,6]. Higher levels of MPs were found in maternal blood in association with preeclampsia (PE), and placental MPs were shown to promote endothelial and immune cell dysfunction *in vitro* [1,2,4,7,8]. MPs can be isolated *in vitro* by mechanical means, during placental perfusion, and from explant culture media following centrifugation at g forces ranging from10,000 to 150,000 [3,4,6]. They range in size from approximately 0.1 to 2 μm, and ultrastructural examination as well as presence of high levels of placental alkaline phosphatase expression confirmed their plasma membrane origin [3,6,9]. To date, studies of placental MPs have largely focused on examination of their levels in maternal blood in combination with changes in maternal blood pressure in normal pregnancy and PE with limited attention to their protein composition [1,2,4,7,10]. Elevated release of several placental proteins is suggested to play an important role in the pathophysiology of PE [6,9]. They include the soluble form of the fms-like tyrosine kinase (sFlt-1) which binds vascular endothelial growth factor (VEGF) and placental growth factor (PlGF), as well as soluble endoglin (sEng) which binds transforming growth factor (TGF)-β [11–17]. These interactions prevent association of these angiogenic factors with their cognate membrane receptors thereby blocking function [11–17]. Increases in maternal serum concentrations of sFlt-1 and sEng along with decreases in PlGF is predictive of PE [12–15,17]. *In vitro* studies suggest that the placental syncytium is a likely source of elevated sFlt-1 and sEng in PE [11–13,16,17]. In addition, PE is also known to be associated with an increased PAI-1/PAI-2 ratio in maternal blood

☆ These studies were supported in part through NIH ARRA Funding, Grant HD33909-13 (S.G.), and Grant 32-061771.00 from the Swiss National Research Foundation (H.S.).

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doi:10.1016/j.placenta.2010.10.011

[18]. Since PAI-1 is a major inhibitor of fibrinolysis through its inhibition of tissue-type plasminogen activator [19], it has been suggested that increased production of syncytial PAI-1 noted in PE may be responsible for aberrantly high levels of fibrin deposition in the intervillous space and placental infarction observed in these in pregnancies [20].

Dual (maternal + fetal) perfusion of a single human placental cotyledon has been used as a physiological model to examine the release and transplacental transfer of compounds at the maternal–fetal interface [21,22]. The maternal compartment, perfused through cannulae inserted directly into the intervillous space, simulates *in vivo* processes whereby syncytiotrophoblasts release proteins to maternal blood [3,23]. Using this system, we previously reported that the PAI-1/PAI-2 ratio in maternal perfusate increases between 1 and 7 h of perfusion [24], and the presence of a reactive oxygen species-generating system (i.e. xanthine/xanthine oxidase) increased the release of MPs and cytokines to the maternal perfusate, enhanced release of 8-iso-PGF2$\alpha$ to the fetal perfusate, and increased IL-1$\beta$ expression in placental tissue, most likely in Hofbauer cells [23]. These observed changes are similar to the increased expression of markers of oxidative stress noted in preeclamptic placentas [25], and suggest that dual perfusion is of specific utility for studying placental pathophysiology in PE.

The objective of this paper was to determine whether MPs released to maternal perfusate during dual perfusion contain factors previously demonstrated to regulate angiogenic and fibrinolytic pathways associated with placental pathophysiology in PE. ELISA, enzymatic assays, and flow cytometry were used to characterize protein expression in MPs.

## 2. Materials and methods

### 2.1. Placental perfusion

Dual *in vitro* perfusion of an isolated cotyledon of a human term placenta was performed as we have previously described [23,24]. Areas of placenta free from fibrin and where the maternal and fetal sides were determined to be intact based on gross morphological examination were used for perfusion. The interval between delivery of the placenta and the start of perfusion was approximately 20–30 min. The perfusion medium consisted of NCTC-135 tissue culture medium with the previously described supplements [23,24]. For both maternal and fetal circuits, 150 ml of perfusate was recirculated with continuous equilibration with air/5% $CO_2$ on the maternal side and 95% $N_2$/5% $CO_2$ on the fetal side. Gas equilibration was carried out using a Silox-S (0.3) oxygenator (Senko Medical Instrument Co., Tokyo, Japan) integrated into both closed circuits ensuring stable $PO_2$ in perfusion reservoirs. Hydrostatic pressure, recorded on line in the fetal arterial branch, varied between 15 and 30 mm Hg in the different experiments. Following initial stabilization, this value remained constant throughout a given perfusion. Flow rates in the maternal and fetal circuits were 12 and 6 ml/min, respectively. After establishing the fetal circuit by catheterization of chorionic plate vessels, and placement of four maternal side catheters providing access to the intervillous space, both compartments were flushed with perfusate for 30 min. After closure of both circuits, buffer was recycled for up to 7 h with changes at 1 and 3 h. These procedures were carried out in 5 independent perfusions.

### 2.2. Extraction of tissue

One-gram tissue specimens obtained from perfused cotyledons at the end of the experiment were compared to unperfused control tissues taken at the start of the perfusion. Tissues were then homogenized in 3 ml of Earle's buffer, centrifuged (14,500×g, 10 min), and aliquots of the supernatant were frozen.

### 2.3. Isolation of MPs

Maternal perfusates at the indicated time points were centrifuged twice at 1500×g for 10 min, and the supernatant was stored at −20 °C. The 1500×g supernatants were then centrifuged at 10,000×g, pellets were washed with PBS and recentrifuged at 10,000×g. The 10,000×g supernatant was centrifuged at 150,000×g, this supernatant was saved, the pellet was washed with PBS, and was centrifuged again at 150,000×g. The final 10,000 and 150,000×g pellets, (designated as 10 K and 150 K MPs, respectively) were resuspended in 300 μl PBS and stored at −80 °C.

### 2.4. Alkaline phosphatase assay

For analysis, MPs were solubilized by incubation at 4 °C for 2 h on a shaker in PBS containing 1% Triton X-100 detergent and a buffer supplemented with 50 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 1% Triton X-100, 10% glycerol, and additional protease and phosphatase inhibitors [1 mM phenylmethylsulfonyl fluoride, 20 mM β-glycerophosphate, 8 mM sodium pyrophosphate, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, and 1 μg/ml aprotinin (Roche Molecular Biochemicals, Indianapolis, IN)]. Samples were then centrifuged at 14,000×g for 30 min and the supernatant was saved for further analysis. For analysis, cleavage of p-nitrophenylphosphate (pNPP) was monitored using an alkaline phosphatase assay kit obtained from AnaSpec Corp (San José, CA). The activity in MP fractions was compared to whole placental tissue homogenates collected before and after perfusion. The sensitivity of the assay was 0.01 ng alkaline phosphatase per ml. Alkaline phosphatase activity in placental tissue extracts and MPs was normalized to total protein using the DC Protein Assay from Bio-Rad Laboratories (Hercules, CA).

### 2.5. PAGE

Electrophoretic separation of extracts of whole placental tissue and MPs was carried out using 4–15% Tris–HCl polyacrylamide protein gels as we have previously described [26]. Visualization of proteins was performed using Imperial™ Protein Stain according to the manufacturer's instructions (Pierce, Rockford, IL).

### 2.6. ELISAs

Levels of total PAI-1 and PAI-2 in the maternal perfusion media and in solubilized MPs were measured by ELISA (catalogue numbers 821 and 823) according to information provided by the manufacturer (American Diagnostica, Stamford, CT). Levels of Eng and sFlt-1 were determined using ELISAs from R&D systems (Minneapolis, MN) (catalogue numbers DVR100B and DNDG00). It is important to recognize that the ELISA used in this study to measure sFlt-1 does not measure membrane Flt-1, since the antibody detects only unique amino acid sequences present in sFlt-1 which arise from alternative splicing of FLT-1 gene products. In contrast, the Eng ELISA recognizes both membrane Eng as well as sEng, as sEng is suggested to arise from cleavage of the membrane-associated form [27].

### 2.7. Flow cytometry

Ten K MPs (2 μg of protein) were incubated with and without 1 μg/ml FITC-Annexin V (R&D Systems) and flow cytometry was carried out (see below) to assess MP purity. Annexin V binds to all classes of MPs regardless of cell type [28]. To estimate the size of MPs, forward scatter patterns were compared to those obtained with beads of known diameter (0.3, 0.6, and 1.1 μm) purchased from Sigma–Aldrich (St. Louis, MO, catalogue numbers LB3, LB6, and LB11, respectively). To obtain cell surface levels of PAI-1, PAI-2, Flt-1, and Eng, 10 K MPs (2 μg) were resuspended in 40 μl of 1%BSA/PBS containing 1 μg human IgG, and were incubated for 15 min at room temperature. Ten μl of FITC or phycoerythrin conjugated primary antibodies and IgG isotype controls were then added and incubation was continued for 45 min at 4 °C (see Table 1 for antibody information). Note, that anti-Eng and anti-Flt-1 antibodies used in flow cytometry were raised against the membrane-associated forms of these proteins. As described above, no antibody is available which detects sEng and not membrane Eng, and those which detected sFlt-1 were either not suitable for use in flow cytometry according to the manufacturer, or in our hands. MPs were then washed twice using 1%BSA/PBS and were resuspended in 400 μl of PBS. Alternatively, MPs were fixed with 4%PFA/PBS for 10 min at room temperature and then permeabilized using 1% Triton X-100/PBS for 5 min at room temperature before blocking and staining. Solutions containing antibodies conjugated to fluorescent labels were centrifuged at 16,000×g for 30 min at 4 °C to remove any particles prior to incubation with MPs. Results were collected using a BD FACSCalibur flow cytometer with bundled CellQuest software and were analyzed using FlowJo software.

### 2.8. Statistics

Results are expressed as a mean + SE. Analysis of time-dependent changes in protein expression, or changes in levels of a specific protein among several groups at a single time point, was carried out by ANOVA followed by pairwise comparison of results using Student-Newman-Keuls method. SigmaStat software from Jandel Scientific (San Rafael, CA) was used for statistical analysis. For all tests, a $P$ value $<0.05$ was considered significant.

### 2.9. Human subjects

For perfusions, human term placentas ($n = 5$) were obtained from pregnancies with normally grown, singleton fetus delivered by cesarean section at term. Informed consent was certified in writing by each patient at the Inselspital Berne, Switzerland.