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

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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.

Keywords

Placental microparticles; Dual perfusion; Preeclampsia; Flt-1; Endoglin; Plasminogen activators

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

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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 from 10,000 to 150,000 [3,4,6]. They range in size from approximately 0.1 to 2 μ m, 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 [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 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-PGF $_{2\alpha}$ to the fetal perfusate, and increased IL-1 β 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% CO₂ on the maternal side and 95% N₂/5% CO₂ 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 PO₂ 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 50mM 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.

3. Results

3.1. Characteristics of 10 K and 150 K MPs

Alkaline phosphatase activity, a plasma membrane marker, was measured in whole placental homogenates before and after perfusion (Fig.1, Left Panel, two left bars) and in 10 K and 150 K MPs isolated from maternal perfusate after 1, 3, and 7 h of perfusion (Fig. 1, Left Panel, 6 right bars). Note that alkaline phosphatase activity was significantly greater (~12-

fold) in MP fractions compared to homogenates confirming that MPs consist of plasma membrane fragments. This conclusion is also supported by flow cytometry results showing strong FITC-labeled Annexin V binding (i.e. a property of all MPs regardless of cell or tissue origin) to 10 K MPs (solid line, Right Panel); the broken line denotes results obtained with MPs not incubated with Annexin V. Ninety-five percent of the 10 K MPs were found to bind Annexin V. The size of MPs was determined based on flow cytometric comparison with beads of known diameter. The observed size distribution for 10 K MPs were: 0.3–0.6 μm (65–80%), 0.6–1.1 μm (10–30%), and >1.1 μm (<5%). In contrast, >96% of 150 K MPs were between 0.3 and 0.6 μm , indicating an overall smaller size compared to 10 K MPs. PAGE revealed similar patterns of protein expression in 10 K and 150 K MPs (Fig. 1, Bottom Panel). A major protein band was noted in both fractions at approximately 60 kDA, most likely reflecting the presence of placental alkaline phosphatase [10]. A band of similar molecular weight was present in whole placental extracts before perfusion, but was greatly reduced in level after perfusion, suggesting that this protein was shed in MPs.

3.2. Protein composition of perfusates, supernatants, and MPs

ELISA was used to examine the levels of PAI-1, PAI-2, sFlt-1, and Eng in maternal perfusates, 150 K supernatants (i.e. the MP-free soluble fraction obtained following centrifugation of the perfusate at 10 K and then 150 K), 10 K MPs, and 150 K MPs. We noted that PAI-1 levels showed a time-dependent increase in both perfusate and supernatant whereas PAI-2 levels showed time-dependent decreases in these fractions (Fig. 2). Supernatant levels of PAI-1 and PAI-2 were nearly identical to that found in the perfusate for each of these respective proteins. For 10 K MPs both PAI-1 and PAI-2 expression showed a time-dependent increase, no change was noted in PAI-1 levels in 150 K MPs, and a time-dependent decrease in PAI-2 was seen in 150 K MPs, although this effect did not reach statistical significance. Of note, PAI-2 levels in MPs were 70–200-fold that observed for PAI-1.

Relatively low levels of sFlt-1 were found in the four fractions, and no significant time-dependent changes were noted (Fig. 3 Top Panels). Of particular note, levels of Eng in perfusate and 150 K supernatant fraction were relatively low, but unlike the 3 other proteins studied, levels of Eng in the supernatant fraction were reduced by approximately 60% compared to the perfusate (Fig. 3, Bottom Panels). A time-dependent 2.5 fold decrease in Eng levels in 150K MPs was noted, no time-dependent changes in Eng levels were noted for the 3 other fractions. Interestingly, levels of Eng in 10 K and 150 K MPs were approximately 3000-, 400-, and 3-fold that noted for sFlt-1, PAI-1, and PAI-2, respectively. The finding of extremely high levels of Eng in MPs, and the observation that Eng levels in the supernatant fraction were less than that of perfusate, indicates that MPs account for a significant portion of Eng released by placenta to maternal perfusate.

3.2.1. Measurement of MP-associated PAI-1, PAI-2, Flt-1, and Eng by flow cytometry—Flow cytometry was carried out to assess the expression and topographical localization of PAI-1, PAI-2, Flt-1, and Eng in MPs. Only 10 K MPs were used in flow cytometry studies as proteins in 150 K MPs could not be reliably detected by this methodology. For each of the proteins studied the number of positive events was calculated by comparison of signals obtained with protein-specific antibody (solid lines) compared to isotype-matched control antibody (broken lines) (Figs. 4 and 5). Please note, that in Panels 4A, 4C, 5A, and 5C flow cytometry was carried out in permeabilized MPs, whereas in Panels 4B, 4D, 5B, and 5D flow cytometry was performed with non-permeabilized MPs (i.e. surface binding was measured). It is of note that flow cytometric analysis of MPs revealed that both PAI-2 and Eng, those proteins expressed at highest levels in MPs by ELISA, showed a significant increase in fluorescence intensity in the presence of protein-specific

antibody both with and without solubilization (Figs. 4 and 5). Based on comparison to the isotype-matched control, the percentage of positive signals for PAI-2 was 96% and 95% with and without permeabilization, respectively; the values for Eng were 94% and 93%, respectively. Conversely, flow cytometry revealed essentially no increase in peak fluorescence intensity in the presence of PAI-1-specific antibody (7% positive events with and without permeabilization) and a relatively small right-shift in fluorescence intensity was noted in the presence of Flt-1 antibody (38% positive events with and without permeabilization). Note that Flt-1, not sFlt-1 levels were measured by flow cytometry. The use of an sFlt-1 antibody did not reveal a protein-specific right shift in fluorescence intensity (not shown). Taken together, these results are consistent with our ELISA data and suggest that surface-associated forms of PAI-2 and Eng are major components of MPs.

4. Discussion

For several years it has been known that MPs are shed from the syncytial surface of placenta to maternal blood [1–4]. This process occurs during uncomplicated pregnancy, and is more dramatic in pregnancies complicated by preeclampsia [4,6]. Although early-onset severe PE is often associated with intrauterine growth restriction (IUGR), ELISA revealed excess shedding of MPs in pregnancies with preterm PE but not normotensive preterm IUGR [2], thus indicating a specific role of MPs in the pathophysiology of PE. It has been suggested that increased release of MPs in PE may be due to elevated levels of syncytial apoptosis due to hypoxia and or hypoxia/reperfusion at this site [5,6]. Since systemic activation of maternal endothelium is considered to play a key role in the pathophysiology of PE [29], several groups have examined the effects of MPs, generated in the laboratory, on endothelial cell functions [1,3,7,30]. These studies, using human umbilical vein endothelial cells as well as dissected maternal subcutaneous arteries, demonstrated that MPs reduce cellular proliferation and function, and depending on the source of MPs, promote apoptosis and/or cell damage [1,3,7,30]. It has also been shown that MPs activate neutrophils based on superoxide and cytokine production, and promote their formation of extracellular DNA lattices [1,7]. More recently, an activation of monocytes with an increased expression of the adhesion molecule CD54 and stimulation of the release of the pro-inflammatory cytokines IL-8, IL-6, and IL-1 β by MPs has been shown [8].

In addition to investigations into the role of MPs in PE, there is a well-described connection between the anti-angiogenic factors, sFlt-1 and sEng, and the pathophysiology of PE [13–15]. These proteins block the angiogenic actions of VEGF and PlGF, as well as TGF- β , respectively, are made by the placenta, and increase in concentration in maternal sera prior to the onset of proteinuria and hypertension in PE [13,14]. In addition, it has been demonstrated that over-expression of sFlt-1 and sEng in pregnant rats causes preeclampsia-like symptoms including the development of glomerular endotheliosis [15,27]. PE is also known to be a prothrombotic, anti-fibrinolytic maternal state with an elevated PAI-1/PAI-2 ratio in maternal blood [18]. PAI-1 is the major inhibitor of fibrinolysis through its suppression of tissue-type plasminogen activator function [19]. Although the placenta expresses extremely high levels of PAI-2 [31], its overall function is less clear as it possesses only one-one thousandth the potency of PAI-1 in the inhibition of fibrinolysis [19]. Alterations in syncytial PAI-1 and PAI-2 synthesis are associated with excessive placental fibrin deposition and infarction in PE [20]. Thus, in light of the importance of MPs, angiogenesis, and fibrinolysis in the pathophysiology of PE, the goal of the current investigation was to examine the expression PAI-1, PAI-2, sFlt-1, and Eng in MPs.

In the current study, dual placental perfusion was chosen as the method to obtain MPs based on its overall attribute of preserving tissue integrity as well the specific observation that MPs released to the maternal perfusate may be more “physiological” in nature compared to those

generated by mechanical disruption of villous tissue [3,8,22]. It is of note that ELISA analysis was carried out in MPs first solubilized by treatment with detergent, thereby releasing both membrane-associated proteins as well those inside the MP. Thus, although MP-associated proteins are released to maternal perfusate (and maternal blood *in vivo*), they are distinct from truly soluble non-membrane associated factors. Our initial results using ELISA showed that both 10 K and 150 K MPs expressed high levels of alkaline phosphatase activity suggesting both are derived from the syncytial plasma membrane. The plasma membrane derivation of MPs was also supported by flow cytometry which revealed high levels of Annexin V binding in 10 K MPs, a hallmark of plasma membrane-derived MPs regardless of tissue or cellular source [28]. In addition, since Annexin V binding and Eng expression are localized to the syncytial surface *in vivo* [32,33], our flow cytometric results showing high levels of Annexin V binding and Eng expression suggest that MPs released during perfusion maintain the sidedness of intact tissue.

Initial comparisons by PAGE and flow cytometric analyses revealed that 10 K MPs were larger than 150 K MPs, but overall patterns of protein expression were similar. It is important to note that flow cytometric analysis of protein expression in 10 K MPs was based on less than 40% of these particles as approximately 10–35% were in the detectable size range (i.e. 0.6–1.1 μm and >1.1 μm). More detailed analysis by ELISA revealed both similarities and differences in the composition and release of 10 K and 150 K MPs, suggesting that they are two distinct populations. Although the relative levels of PAI-1, PAI-2, sFlt-1, and Eng were similar in both MP fractions, levels of PAI-1 and PAI-2 increased in 10 K MPs during perfusion but not in 150 K MPs. In contrast, levels of sFlt-1 and Eng levels in 10 K MPs did not change during perfusion whereas levels in 150 K MPs decreased, most likely reflecting a time-dependent decrease in total protein in the 150 K fraction (not shown). For both MP fractions the relative level of expression of the four proteins was Eng>PAI-2» PAI-1>sFlt-1. It is of note that levels of PAI-1 and PAI-2 in maternal perfusate increased and decreased between 1 and 7 h of perfusion, respectively. This supports our previous finding [24] and most likely indicates the placenta remains in a relatively hypoxic state following its delivery despite efforts to maintain high levels of oxygenation during perfusion. In contrast, no significant time-dependent changes in levels of sFlt-1 or Eng in maternal perfusate were noted. Whereas levels of PAI-1, PAI-2, and sFlt-1 in the 150 K supernatant were identical to that observed in the perfusate, supernatant levels of Eng were approximately one-half to one-third that found in the perfusate. This indicates that the majority of Eng released to the maternal perfusate is MP-associated, and suggests that a significant fraction of sEng in maternal sera may be derived from placental MPs.

Flow cytometry was carried out to localize PAI-1, PAI-2, Eng, and Flt-1 proteins in 10 K MPs. Attempts to localize these proteins in 150 K MPs were not successful as their smaller size was most likely incompatible with this method of analysis. Two important conclusions were obtained from these experiments: the overall expression patterns of these proteins studied supported that found by ELISA (i.e. that expression of Eng and PAI-2 exceeded that of PAI-1 and Flt-1) and; virtually all of the Eng and PAI-2 was detected at the surface of MPs. This later finding may have important implications in the pathophysiology of PE. Soluble Eng exerts its anti-angiogenic actions by binding TGF- β thereby preventing its interaction with membrane Eng [15,27]. Our results indicate that Eng, found on the surface of MPs, may similarly bind to TGF- β and block its biological function. The finding of PAI-2 on the surface of MPs was unexpected as it was previously localized to the syncytial cytoplasm [34]. However, PAI-2 associated with cell-surface urokinase-type plasminogen activator is described [35,36], suggesting its presence on the surface of MPs reflects a physiological or pathological response during perfusion. The significance of MP-associated PAI-2 is less clear. At high concentrations it would be anti-fibrinolytic, potentially leading to excessive fibrin deposition in the intervillous space and placental infarction noted in PE

[37]. PAI-2 also blocks TNF- α -mediated apoptosis [38], suggesting a role in regulating cell death. Since the syncytium is bathed by maternal blood, maternal immune cells in the intervillous space may be targets of placental MPs. Smaller MPs, not cleared by maternal lung, may promote more systemic effects on the maternal endothelium. It is also important to consider MPs derived from non-placental cellular sources in the pathophysiology of PE. Previous studies indicate that platelet-derived MPs may play an important role in regulating vascular function during pregnancy [39,40].

We conclude that placental MPs are a rich source of proteins which may alter the angiogenic and fibrinolytic balance at the maternal–fetal interface, which may ultimately maintain a physiological balance of these processes in normal pregnancy or promote deleterious actions in the maternal syndrome of PE.

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Fig. 1.

Characteristics of MPs isolated from maternal perfusate. In the Left Panel alkaline phosphatase activity was measured in whole placental homogenates before and after perfusion (2 Left Bars), and in 10 K (3 Middle Bars) and 150 K (3 Right Bars) MPs obtained from the maternal perfusate at the indicated time of perfusion. Results are presented in log scale as a mean $\pm$ SE, and the level of statistical significance is indicated by the connecting lines at the top of the figure. Flow cytometric analysis of 10 K MPs, isolated from a 3 h perfusion sample, in the absence (broken line) and presence (solid line) of FITC-labeled Annexin V is shown in the Right Panel. In the Bottom Panel PAGE analysis was carried out for the following samples; Lane 1, 10 K MPs; Lane 2, 150 K MPs; Lane 3, whole placental

tissue before perfusion; Lane 4, tissue after perfusion; Lane 5, protein standards of molecular weights indicated at the right of the panel. The arrow refers to a protein band of approximately 60 kDa. MPs from 5 different perfusions were used for alkaline phosphatase analysis, and a representative experiment of 3 identically conducted ones is shown for Annexin V binding and PAGE.

Fig. 2.

Analysis of PAI-1 and PAI-2 levels in maternal perfusate, supernatant, and MPs. Levels of PAI-1 and PAI-2 in maternal perfusate, the final MP-free 150K supernatant, and in 10 K and 150 K MPs, were determined by ELISA. Results are expressed as a mean + SE obtained from 5 independent perfusions, and levels of statistical significance are shown by interconnecting lines at the top of the individual panels.

Fig. 3.

Levels of sFlt-1 and Eng in maternal perfusate, supernatant, and MPs. The concentration of sFlt-1 and Eng in maternal perfusate, the MP-free supernatant, and in 10 K and 150 K MPs, was quantitated by ELISA. Results are expressed as a mean + SE obtained from 5 independent experiments, and the level of statistical significance is shown by interconnecting lines at the top of the individual panels.

Fig. 4.

Flow cytometric analysis of PAI-1 and PAI-2 expression in 10 K MPs. Flow cytometric analysis of PAI-1 and PAI-2 was carried out in permeabilized (Panels A, C) or non-permeabilized (Panels B, D) 10 K MPs. Experiments were performed in the presence of protein-specific antibody (solid line) or isotype-matched control antibody (broken line). Representative results from 1 of 3 independent experiments are shown.

Fig. 5.

Flow cytometric analysis of Flt-1 and Eng in 10 K MPs. Flow cytometry was carried out to assess levels of Flt-1 and Eng in permeabilized (Panels A, C) or non-permeabilized (Panels B, D) 10 K MPs. Experiments were performed in the presence of protein-specific antibody (solid line) or isotype-matched control antibody (broken line). Representative results from 1 of 3 independent experiments are shown.

Table 1

Specificities and sources of monoclonal antibodies used for flow cytometry.

Antibody	mAb/clone	Isotype	Immunogen	Source
Endoglin-FITC	166707	Mouse IgG1	Recombinant human Endoglin extracellular domain (rhEndoglin; aa 25–587)	R&D Systems
FIt-1-PE	49560	Mouse IgG1	Recombinant human VEGF R1 extracellular domain (rhVEGF R1; aa 27–758)	R&D Systems
PAI-1	3780	Mouse IgG2b	Peptide (aa 110–145)	American Diagnostica
PAI-2	3750	Mouse IgG2a	Purified PAI-2 from placental tissue	American Diagnostica
Control-FITC	11711	Mouse IgG1	N/A	R&D Systems
Control-PE	11711	Mouse IgG1	N/A	R&D Systems
Control for PAI-1	133303	Mouse IgG2b	N/A	R&D Systems
Control for PAI-2	133304	Mouse IgG2a	N/A	R&D Systems