Plasma from preeclamptic women activates endothelial cells via monocyte activation in vitro

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ABSTRACT

In this study we tested whether plasma from preeclamptic women contains factors that can activate endothelial cells in the presence of monocytes in vitro. Plasma from preeclamptic women (n=6), healthy pregnant women (n=6) and nonpregnant women (n=6) was incubated with mono-cultures and co-cultures of human umbilical vein endothelial cells (HUVEC) and monomac-6 monocytes. Reactive oxygen species (ROS) production and ICAM-1 expression were measured using flow cytometry. Whether scavenging of ROS by superoxide dismutase and catalase inhibited HUVEC ICAM-1 expression was also investigated. We found that in HUVEC co-cultured with monomac-6 cells but not in HUVEC cultured alone, ICAM-1 was upregulated after incubation with plasma from preeclamptic women but not plasma from non-pregnant women. Also in co-cultures, monomac-6 ICAM-1 was upregulated by plasma from preeclamptic women, while in both mono- and co-cultures monomac-6 ROS production was upregulated by plasma from pregnant and preeclamptic women, compared with plasma from non-pregnant women. Scavenging of ROS by superoxide dismutase and catalase resulted in a further upregulation of HUVEC ICAM-1 after incubation with plasma from preeclamptic women, compared with incubation without superoxide dismutase and catalase. These results show that endothelial cells in vitro are activated by plasma of preeclamptic women only if they are co-cultured with monocytes. This upregulation appeared not to be due to extracellular ROS production by monocytes or HUVEC, pointing to involvement of other mechanisms. Our data suggest that plasma of preeclamptic women activates monocytes, and that these monocytes subsequently activate endothelial cells.

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1. Introduction

Preeclampsia is characterized by hypertension and proteinuria occurring in the second half of pregnancy and is a leading cause of morbidity and mortality in pregnancy. Although the pathogenesis of the disease is not completely understood, it is accepted that a generalized inflammatory response, including activated endothelial cells, plays an important role (Austgulen et al., 1997; Faas and Schuiling, 2001; Sacks et al., 1998). Various studies have shown activation of endothelial cells in preeclampsia by indirect measures, such as increased soluble ICAM-1, soluble VCAM-1, soluble E-selectin and von Willebrand factor (Austgulen et al., 1997; Coata et al., 2002; Deng et al., 1994). This activation of endothelial cells may be restricted...
to specialized vascular beds, as found in the kidney or in the liver (Donker et al., 2005b; Lyall et al., 1999). There is also direct evidence for the activation of inflammatory cells in preeclampsia (Bowen et al., 2001; Gervasi et al., 2001; Holthe et al., 2004; Sacks et al., 1998). For instance upregulation of the activation marker CD11b, increased production of reactive oxygen species (ROS) and down-regulation of L-selectin were shown on both monocytes and granulocytes of preeclamptic women as compared with healthy pregnant women (Sacks et al., 1998). During normal pregnancy, the inflammatory response is also activated, as indicated by phenotypically activated leukocytes (Luppi et al., 2002; Sacks et al., 1998) and an altered monocyte cytokine production as compared with non-pregnant women (Veenstra van Nieuwenhoven et al., 2003).

The factor(s) responsible for activating the inflammatory response and endothelial cells during preeclampsia are unknown. During preeclampsia reduced vascular trophoblast invasion and insufficient remodeling of the spiral arteries result in abnormal placentation and placental underperfusion (Borzychowski et al., 2006). This may lead to the release of factors from the dysfunctional placenta. These factors may then systemically activate inflammatory cells, such as monocytes, granulocytes and endothelial cells. Many candidate placental factors have been proposed, such as cytokines (Rusterholz et al., 2007) and syncytiotrophoblast microvillous membrane fragments (Germain et al., 2007). However, nonplacental factors may also be involved, for instance circulating microparticles released from other cells (Lok et al., 2006). In two recent studies we tested whether plasma from pregnant and preeclamptic women can activate monocytes or endothelial cells in vitro (Donker et al., 2005a; Faas et al., 2008). We found that plasma from both pregnant women and preeclamptic patients can activate monocytes in vitro (Faas et al., 2008), but these plasma samples did not activate endothelial cells directly (Donker et al., 2005a).

In the present study we therefore tested the hypothesis that plasma of preeclamptic women can activate monocytes, which in turn activate endothelial cells. To test this hypothesis we used mono-cultures and co-cultures of monocytes and endothelial cells and we incubated these cultures with plasma from preeclamptic women, gestational age-matched healthy pregnant women and age-matched healthy non-pregnant women. In accordance with our previous study (Faas et al., 2008), we measured the activation of monocytes and endothelial cells using ICAM-1 expression and reactive oxygen species production (ROS).

2. Materials and methods

2.1. Patients

This study was approved by the Medical Ethics Committee of the University Medical Center Groningen. Written informed consent was obtained from all participants. Preeclampsia was defined according to the International Society for the Study of Hypertension in Pregnancy (diastolic blood pressure: >90 mm Hg on two or more consecutive occasions, and proteinuria of more than 300 mg/24 h, developing after 20 weeks of gestation) (Table 1). Exclusion criteria were pre-existing hypertension, diabetes mellitus, vasculitis, chronic renal disease, autoimmune disease or malignancy and recent trauma or surgery. All preeclamptic patients (n = 6) had early-onset disease and at the time of blood sampling had not been treated with corticosteroids.

Controls were healthy pregnant women (n = 6), matched for age (±6 years) and gestational age at blood sampling (±2 weeks). Nonpregnant women (n = 6) recruited from our hospital staff were matched for age. For both the normal pregnancy and the preeclamptic group only singleton pregnancies were included (see Table 1).

2.2. Collection and preparation of plasma samples

Blood samples of pregnant and preeclamptic women were collected from the antecubital vein during routine blood sampling into lithium heparin tubes (Venoject, Terumo Europe NV, Leuven, Belgium), immediately stored at 4 °C and centrifuged within 1 h (130 g for 10 min at 4 °C followed by 700 × g for 10 min) and plasma was frozen at −80 °C until further use. Care was taken that in each separate experiment, mono- and co-cultures were incubated with plasma from a preeclamptic patient, from a healthy pregnant woman (age- and gestational age-matched), and a nonpregnant woman (age-matched).

2.3. Mono-cultures

We used the monocyte cell line (monomac-6 (MM6)), which is a monocytic cell line, showing phenotypic and functional features of mature monocytes (Ziegler-Heitbrock et al., 1988). The reason for using this cell line, rather than isolated peripheral blood monocytes is the fact that isolation of monocytes from peripheral blood in itself already results in activation of the monocytes (Macey et al., 1995; Sacks et al., 1997). The MM6 cell line was cultured as described before (Faas et al., 2008). Three days before the start of the experiments, MM6 were transferred to endothelial cell medium (see below) to ensure that all experiments were performed in the same medium. HUVEC were obtained from the Endothelial Cell Facility of the University Medical Center Groningen as described before (Donker et al., 2005a). In brief, primary isolates of two umbilical cords were mixed and subsequently cultured on 1% gelatin precoated 25 cm2 tissue culture flasks (Corning, Costar, Netherlands) at 37 °C in 5% CO2 and 100% humidity. The culture medium consisted of RPMI 1640 supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS), 2 mM l-glutamine, 5 U/ml heparin, 100 IE/ml penicillin, 100 μg/ml streptomycin, and 50 μg/ml endothelial cell growth factor supplement extracted from bovine brain. The experiments were started with confluent monolayers of HUVEC in 12-well plates (45,000–50,000 cells/cm2; 250,000 cells per well).

2.4. Co-culture of MM6 cells and HUVEC

Two milliliters of MM6 (125,000 cells/mL) was added to the confluent layers of HUVEC in the 12-well plates. For
mono-cultures, 2 mL of MM6 (125,000 cells/mL) was added to empty wells, and 2 mL of only endothelial cell medium was added to HUVEC containing wells (250,000 cells/well). Cultures were incubated for 24 h before the start of the experiment at 37 °C, 5% CO₂.

2.5. Experimental setup

We first tested the effect of co-culturing and LPS stimulation on MM6 and HUVEC ICAM-1 expression and ROS production. Therefore, to test the effect of 24 h co-culturing on MM6 and HUVEC was assessed by measuring MM6 and HUVEC ICAM-1 expression and ROS production using flow cytometry as described below (n=5). To test the effect of LPS stimulation after 24 h of co-culture, mono- and co-cultures were incubated with 15% FCS with or without LPS (2 μg/mL) for 4 h (ROS production) or 24 h (ICAM-1 expression) and ICAM-1 expression and ROS production were measured as described below (n=5).

After these initial experiments, which showed that ICAM-1 and ROS are good markers of endothelial cell and monocyte activation in the present model, we tested the effect of plasma from the 3 groups of women on ICAM-1 expression and ROS production.

In the first experiment we investigated the effects of plasma from the 3 groups of women on ICAM-1 expression (protein and mRNA) of mono-cultures and co-cultures. Therefore, after the initial 24 h of co-culture, cultures were incubated in endothelial cell medium with 15% plasma from nonpregnant (n=6), pregnant (n=6) and preeclamptic women (n=6) or 15% FCS (as control) for 4, 16 and 24 h. After incubation, 70% of the cells were used to measure ICAM-1 mRNA as described below. The remaining 30% was used to measure ICAM-1 protein expression using flow cytometry as described below.

In the second experiment we studied the effect of plasma from nonpregnant, pregnant and preeclamptic women on ROS production of mono-cultures and co-cultures. Cultures were incubated in endothelial cell medium with 15% plasma of either pregnant (n=6), preeclamptic (n=6) or non-pregnant women (n=6) or 15% FCS (as control) for 4, 16 or 24 h. ROS production was subsequently measured as described below.

In the last experiment we studied the effect of extracellular ROS on ICAM-1 expression in MM6/HUVEC co-cultures after incubation with plasma of pregnant, preeclamptic and non-pregnant women. Co-cultures were incubated in endothelial cell medium with 15% plasma of pregnant (n=4), preeclamptic (n=4) or non-pregnant women (n=4) for 24 h in the presence or absence of SOD (70 U/mL) and catalase (70 U/mL). After 24 h incubation, the cells were prepared for measuring HUVEC ICAM-1 protein expression using flow cytometry as described below.

2.6. Flow cytometry

**ICAM-1 expression:** Following incubation, cells were collected from the wells in 5 mL tubes following trypsin/EDTA treatment according to standard procedures. Cells from each well were separated into two aliquots. Both aliquots were incubated with anti-CD45-PE to discriminate MM6 cells (CD45⁺) from HUVEC (CD45⁻) for 30 min at room temperature in the dark. After washing with phosphate buffered saline (PBS) with 0.5% BSA and 0.1% sodium azide (washing buffer), cells were fixed with paraformaldehyde (PFA; 0.5% PFA in PBS) for 30 min. After washing, cells were permeabilized with ice-cold 0.1% Triton X-100 in PBS for 10 min with the fluorogenic substrate dihydrorhodamine 123 according to the manufacturer’s protocol. The
reaction was subsequently stopped and cells were fixed by adding 2 mL lysing solution for 20 min. Cells were then detached from the wells by trypsin/EDTA-treatment and collected in 5 mL tubes. After centrifugation (600 × g, 10 min), the supernatant was discarded and PE-labeled anti-CD45 (IQProducts, Groningen, The Netherlands) was added to the cells for 30 min. The cells were washed with washing buffer and centrifuged (600 × g, 10 min) and 150 µl washing buffer was added to the cells. Cells were kept on ice and measured by flow cytometry within 30 min. ROS-positive cells (both MM6 cells and HUVEC) can easily be identified as a clear population in the sidescatter-FITC plot. Percentage positive cells as well as mean channel brightness of the positive cells (as a measure of total amount of oxygen free radicals produced) was calculated using Winlist 6.0 according to the protocol described in the kit.

2.7. RNA isolation

The cells used for mRNA measurement were labeled with CD45-PE. Therefore, cells were collected in a 5 mL tube and spun down at 600 × g for 10 min. After incubation with CD45-PE for 30 min at room temperature in the dark, the cells were washed twice with washing buffer and sorted into CD45+ (MM6) and CD45− (HUVEC) cells using a fluorescence-activated cell sorter (FACS, MoFlo flow cytometer, Becton Dickinson). RNA was isolated from the CD45+ and CD45− cells using the absolutely RNA® Microprep kit (Stratagene) according to the manufacturer’s protocol. In brief, after cell lysis with the lysis buffer, the lysed cell solution is mixed with ethanol and applied to micro-spin columns to which RNA binds. DNA contamination was removed by incubation with Dnase-I directly on the column. The columns were then washed twice to remove contaminants and total RNA was recovered in 30 µl of elution buffer. Quality of RNA was checked by standard gel electrophoresis and quantification was performed using a Nanodrop. Total RNA was consistently found to be pure and intact.

2.8. Quantitative real-time RT-PCR

mRNA was converted into first strand cDNA using Superscript III RNase H-Reverse Transcripase (Invitrogen, Breda, The Netherlands) with oligo-dT-primers (Promega, Leiden, The Netherlands) and RNase inhibitor (RNaseOUT™, Invitrogen). After the RT-reaction, the cDNA was diluted to 4 ng/µl. Real-time PCR amplifications were performed on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Applera Nederland, Nieuwekerk aan de IJssel, The Netherlands). We used specific primer sets for ICAM-1 and β-actin as described before (Faas et al., 2008) with Cybr-green (Cybr Green PCR Master mix, Applied Biosystems). Real-time PCR reaction was performed as described before (Faas et al., 2008). Sample cycle threshold (Ct) values were determined from plots of relative fluorescence units (RFU) versus PCR cycle number during exponential amplification to enable sample measurement comparisons. The Ct value for ICAM-1 expression was normalized to β-actin expression (delta

\[ \text{Ct} = \text{Ct(β-actin)} - \text{Ct(ICAM-1)} \]. Delta delta Ct of the various incubation times of plasma from pregnant and preeclamptic women versus plasma from nonpregnant women was evaluated and fold-change versus plasma from nonpregnant women was calculated.

2.9. Statistics

All results are expressed as mean ± standard error of the mean (SEM). For the experiments presented in Fig. 1, in which we tested the effect of co-culturing and LPS stimulation on ICAM-1 protein expression and ROS production, we used Wilcoxon’s Signed Rank test to evaluate the differences between mono- and co-culture. In the experiments in which ICAM-1 protein and mRNA expression and ROS production were measured, effects of the culture condition (co- or monoculture) and effects of pregnant state (non-pregnant, pregnant or preeclamptic) were tested using two-way ANOVA followed by Bonferroni post-tests. In the case of ICAM-1 mRNA expression at 4 and 16 h, we used Wilcoxon’s Signed Rank test to evaluate differences caused by plasma incubation from the three groups of women. In all cases, the significance level was \( p < 0.05 \). In experiments in which plasma from the 3 groups of women were incubated with mono- and co-cultured MM6 and HUVEC, results for nonpregnant women (at each interval) were set at 100%, and the results for pregnant and preeclamptic women are presented as percentages of the results for nonpregnant women at the same intervals.

3. Results

3.1. Effect of co-culturing and LPS stimulation on MM6 and HUVEC ICAM-1 expression and ROS production

Before commencing experiments in which mono- and co-cultures were incubated with plasma, we first co-cultured the MM6 and HUVEC for 24 h in the presence of medium only and tested the effect of co-culturing per se on ICAM-1 expression and ROS production. Although we found no effect of co-culturing on viability of MM6 or HUVEC (results not shown), Fig. 1 (left panels) shows that ICAM-1 expression and ROS production by HUVEC were significantly increased in co-culture as compared with mono-culture.

We next tested the effect of LPS stimulation in mono- and co-cultures on MM6 and HUVEC ICAM-1 expression and ROS production. Mono- and co-cultures were incubated with 15% FCS without LPS, starting after 24 h of co-culture. Incubations of mono- and co-cultures with 15% FCS without LPS did not further affect HUVEC or MM6 ICAM-1 expression or ROS production compared to just 24 h of co-culture (results not shown). However, incubation of mono- and co-cultures with 15% FCS with LPS did not further affect HUVEC or MM6 ICAM-1 expression or ROS production compared to just 24 h of co-culture.
3.2. Experiment 1: ICAM-1 expression in co-cultures and mono-cultures following plasma incubation

Since none of the experiments showed differences between 24 h incubation with plasma of nonpregnant women compared to incubation with FCS, we do not present the results for the control incubation with FCS and in the remainder of Section 3 we present results as percentage of incubation of plasma from nonpregnant women at the same interval.

After co-culture, in HUVEC cells, the plasma of preeclamptic women, but not the plasma of pregnant and/or nonpregnant women, increased the percentage of ICAM-1-expression and increased the intensity of ICAM-1 staining (Fig. 2a and b) after 16 and 24 h of incubation (Bonferroni post-test, \( p < 0.05 \)). ICAM-1 mRNA in co-cultured HUVEC was only detected after 24 h of incubation; at that time point, ICAM-1 mRNA expression was increased after incubation with plasma from pregnant and preeclamptic women as compared with plasma from nonpregnant women (Wilcoxon’s Signed Rank test) (Fig. 2c). This result was only observed in HUVEC co-cultures. In HUVEC monocultures no effect of plasma from pregnant or preeclamptic women on ICAM-1 protein or mRNA expression was seen compared with plasma from nonpregnant women.

In co-cultures of MM6 and HUVEC, plasma from preeclamptic women increased the percentage of ICAM-1-positive MM6 cells after 16 h of culture compared with plasma from nonpregnant and pregnant women (Bonferroni post-test, \( p < 0.05 \)) (Fig. 3a). The intensity of staining for ICAM-1 on MM6 cells was also increased at this time point by plasma of preeclamptic women compared with plasma from nonpregnant or pregnant women (Bonferroni post-test, \( p < 0.05 \)) (Fig. 3b). In mono-cultures of MM6 we found no effect of plasma from pregnant and preeclamptic women on the percentage of ICAM-1-positive cells compared with plasma from nonpregnant women (Fig. 3a). The intensity of staining for ICAM-1 on mono-cultured MM6 was decreased after incubation with plasma from pregnant and preeclamptic women versus plasma of nonpregnant women after 24 h of incubation (Bonferroni post-test, \( p < 0.05 \)) (Fig. 3b).

ICAM-1 mRNA in co-cultured MM6 cells was only detected after 24 h of incubation; no difference in ICAM-1 mRNA expression was observed when comparing incubations with plasma of the 3 groups of women (Fig. 3c). In MM6 mono-cultures ICAM-1 mRNA was upregulated after incubation with plasma from preeclamptic women as compared to incubation with plasma from nonpregnant or pregnant women after 4 h of incubation. After 24 h of incubation both plasma of pregnant and preeclamptic women downregulated ICAM-1 mRNA expression compared to incubation with plasma from nonpregnant women.

3.3. Experiment 2: ROS production in co-cultures and mono-cultures following plasma incubation

For these studies we only show ROS production from HUVEC cells in co-culture, since HUVEC in mono-culture produce barely detectable ROS (Fig. 1). Indeed, two-way ANOVA showed an effect of culturing on all time points. In co-cultures, the percentage of HUVEC producing ROS was significantly increased after 4 h and significantly decreased after 24 h of incubation with plasma from pregnant women compared to plasma from nonpregnant women (Fig. 4, left panel, Bonferroni post-test, \( p < 0.05 \)). Plasma from
ICAM-1 protein and mRNA expression by HUVEC in co-cultures and mono-cultures. (a) Percentage ICAM-1 expressing HUVEC after incubation of HUVEC/MM6 co-cultures (left panel) and HUVEC mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) or preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of non-pregnant plasma. For each time-point, data were analyzed using two-way ANOVA: an effect of pregnancy status ($p < 0.05$) was found at 16 h; an effect of pregnancy status and an interaction between the culture condition and pregnancy status was found at 24 h ($p < 0.05$). (*) Significantly increased vs. non-pregnant plasma ($p < 0.05$, Bonferroni post-test). (a) Significantly increased vs. pregnant plasma ($p < 0.05$, Bonferroni post-test). (b) Mean fluorescent intensity of ICAM-1 on HUVEC after incubation of HUVEC/MM6 co-cultures (left panel) and HUVEC mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) or preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of values with nonpregnant plasma. For each time-point, data were analyzed using two-way ANOVA: an effect of pregnancy status ($p < 0.05$) was found after 16 h of incubation; an effect of pregnancy status and an interaction between the culture condition and pregnancy status was found at 24 h ($p < 0.05$). (*) Significantly increased vs. non-pregnant plasma ($p < 0.05$, Bonferroni post-test). (a) Significantly increased vs. pregnant plasma ($p < 0.05$, Bonferroni post-test). (c) ICAM-1 mRNA expression in HUVEC after incubation of HUVEC/MM6 co-cultures (left panel) and HUVEC mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) and preeclamptic women (dotted bars) for 4, 16 or 24 h. Results are expressed as fold-change from nonpregnant plasma. Two-way ANOVA was performed for the 24 h interval; an effect of pregnancy status ($p < 0.05$) and an interaction between pregnancy status and the culture condition was found ($p < 0.05$). For the other intervals differences between the three groups of plasma were tested using Wilcoxon Signed Rank test. (*) Significantly increased vs. non-pregnant plasma (Bonferroni post-test, $p < 0.05$).
Fig. 3. ICAM-1 protein and mRNA expression on MM6 in co-cultures and mono-cultures. (a) Percentage ICAM-1 expressing MM6 cells after incubation of HUVEC/MM6 co-cultures (left panel) and MM6 mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) and preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of values with nonpregnant plasma. For each time-point, data were analyzed using two-way ANOVA: after 16 h of culture an effect of pregnancy status ($p < 0.05$) and of culture condition ($p < 0.05$) was found. (*) Significantly increased vs. non-pregnant plasma ($p < 0.05$, Bonferroni post-test). (a) Significantly increased vs. pregnant plasma ($p < 0.05$, Bonferroni post-test). (b) Mean fluorescent intensity of ICAM-1 on MM6 after incubation of HUVEC/MM6 co-cultures (left panel) and MM6 mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) or preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of values with nonpregnant plasma. For each time-point, data were analyzed using two-way ANOVA: after 16 h of incubation an effect of pregnancy status ($p < 0.05$) and of culture condition ($p < 0.05$) was found; also an interaction between culture condition and pregnancy status ($p < 0.05$) was found at 24 h. (*) Significantly increased/decreased vs. non-pregnant plasma ($p < 0.05$, Bonferroni post-test). (a) Significantly increased vs. pregnant plasma ($p < 0.05$, Bonferroni post-test). (c) ICAM-1 mRNA expression of MM6 after incubation of HUVEC/MM6 co-cultures (left panel) and MM6 mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) and preeclamptic women (dotted bars) for 4, 16 or 24 h. Results are expressed as fold-change from nonpregnant plasma. Two-way ANOVA was performed for the 24 h interval; an effect of pregnancy status ($p < 0.05$) and an interaction between pregnancy status and culture condition ($p < 0.05$) was found. For the other intervals, differences between the three groups of plasma were tested using Wilcoxon Signed Rank test. (*) Significantly increased vs. pregnant plasma ($p < 0.05$, Bonferroni post-test). (a) Significantly increased vs. non-pregnant plasma ($p < 0.05$, Wilcoxon); (b) Significantly increased vs. pregnant plasma ($p < 0.05$, Wilcoxon).
Fig. 4. ROS production in HUVEC in co-culture. Percentage ROS producing HUVEC (left panel) and ROS staining intensity of HUVEC (right panel) after incubation of HUVEC/MM6 co-cultures with plasma from nonpregnant (open bars), pregnant (black bars) or preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of values with nonpregnant plasma. For each time-point, data were analyzed using two-way ANOVA: for percentage of positive cells an effect of the culture condition \( (p < 0.05) \) and pregnancy status \( (p < 0.05) \) was shown for all time points. Two-way ANOVA also showed an effect of culture condition \( (p < 0.05) \) on the intensity of staining at all time points. (*) Significantly increased/decreased vs. non-pregnant plasma \( (p < 0.05, \text{ Bonferroni post-test}) \). (a) Significantly decreased vs. pregnant plasma \( (p < 0.05, \text{ Bonferroni post-test}) \).

Fig. 5. ROS production in MM6 in co-culture and mono-culture. (a) Percentage ROS producing MM6 cells after incubation of HUVEC/MM6 co-cultures (left panel) and MM6 mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) and preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of nonpregnant plasma. For each time-point, data were analyzed using two-way ANOVA: after 4 h of incubation, an effect of pregnancy status \( (p < 0.05) \) was shown. (*) Significantly increased/decreased vs. non-pregnant plasma \( (p < 0.05, \text{ Bonferroni post-test}) \). (b) Mean fluorescent intensity of ROS in MM6 after incubation of HUVEC/MM6 co-cultures (left panel) and MM6 mono-cultures (right panel) with plasma from nonpregnant (open bars), pregnant (black bars) and preeclamptic women (dotted bars) for 4, 16 or 24 h. Data are expressed as percentage of values with nonpregnant plasma. For each time-point, data were analyzed using two-way ANOVA: after 4 h of incubation, an effect of pregnancy status \( (p < 0.05) \) was shown. (*) Significantly increased/decreased vs. non-pregnant plasma \( (p < 0.05, \text{ Bonferroni post-test}) \).
and endothelial cell activation

4. Discussion

We also showed in the present study that plasma from pregnant and preeclamptic women did not activate HUVEC when cultured in mono-culture. This confirms results from our own laboratory and from others (Donker et al., 2005a; Lorentzen et al., 1991). The present paper shows that HUVEC can be activated by plasma of preeclamptic women when the cells are co-cultured with monocytes. This suggests that in this in vitro experiment, factors in plasma of preeclamptic women activate MM6 cells and these in turn activate HUVEC. We also showed that in the co-culture, HUVEC are not activated through extracellular ROS production by MM6 cells or HUVEC, since scavenging of ROS by SOD and catalase increased rather than decreased HUVEC ICAM-1 expression following incubation with plasma from preeclamptic women.

In this study we used a monocyte cell line (monomac-6) that has characteristics of mature circulating monocytes (Ziegler-Heitbrock et al., 1988). We used HUVEC because these cells are readily available and have been used in many previous studies including our own (Donker et al., 2005a). Furthermore, there are many similarities between HUVEC and human glomerular microvascular endothelial cells (Donker et al., 2005a) as was recently shown by us. Since monocytes and endothelial cells influence each other in co-culture (Noble et al., 1999; Schubert et al., 2008; Tsouknos et al., 2003), we decided to co-culture the cells for 24 h before starting the incubation with plasma. Indeed, just co-culturing MM6 with HUVEC for 24 h without adding the extra plasma resulted in an upregulation of HUVEC ICAM-1 expression and HUVEC ROS production. No further upregulation of ICAM-1 and ROS was observed after 24 h of co-culture, suggesting that the cells adapt to the co-culture conditions. We used ICAM-1 expression and ROS production as markers of activation of MM6 and HUVEC. ICAM-1 is a very good marker of activation for both cell types, as previously shown (Hartmann and Krieg, 1999; Lawson and Wolf, 2009). Additionally, the production of ROS is significantly upregulated after activation of mono-cultures and co-cultures with LPS. In our study mono-cultures of HUVEC did not produce ROS after LPS stimulation. This appears to contradict previous studies showing that LPS induced ROS production in HUVEC (Park et al., 2006; Simon and Fernandez, 2009). Differences may be explained by the stimulation of LPS: we stimulated for 4 h with LPS, while the other studies measured effects at much earlier time points, i.e. within 30 min or 1 h after LPS stimulation. Together these 2 markers are therefore good markers of activation for HUVEC and MM6 cells.

In accordance with previous studies (Donker et al., 2005a; Zhang et al., 2006), we found no difference in HUVEC ICAM-1 expression after incubation of HUVEC mono-cultures with plasma from pregnant women as compared to incubation with plasma from preeclamptic women. In addition, we now show that incubation with plasma from pregnant and/or preeclamptic women does not change HUVEC ICAM-1 expression compared to incubation with plasma from nonpregnant women. These results suggest that plasma from pregnant and preeclamptic women does not directly activate endothelial cells in vitro. In contrast to our study, Takacs et al. (2001) showed increased ICAM-1 expression on endothelial cells in vitro follow-
ing incubation with plasma from preeclamptic women. There are 2 important differences between our study and the study of Takacs et al, which may explain the differences in results: we used plasma from early onset severe preeclamptic women, while Takacs et al. used plasma of late onset severe preeclamptic women. Moreover, we incubated HUVEC with plasma for a maximum of 24 h, while Takacs et al. incubated HUVEC with plasma for 48 h. Comparison between these two studies is difficult because the patient population, the gestational age at blood sampling, the plasma or serum incubation period, the origin of endothelial cells, and the readouts for endothelial activation are all different.

In contrast to mono-cultures, HUVEC were activated when co-cultures of HUVEC and MM6 cells were incubated with plasma from preeclamptic women. After 24 h of incubation, ICAM-1 protein upregulation in co-cultured HUVEC appeared to be induced by increased mRNA expression. However, despite the upregulation of ICAM-1 mRNA after 24 h of incubation with plasma of pregnant women, we did not observe a concomitant upregulation of ICAM-1 protein. Whether this is due to decreased mRNA half-life, increased shedding of ICAM-1 or other mechanisms remains to be established. The mechanism of ICAM-1 upregulation at 16 h remains uncertain as well, since we could not detect ICAM-1 mRNA expression after 16 h of incubation. This was not a technical problem, since RNA isolation and PCR were all done at the same time for all time points of an experiment and RNA was present and intact in all samples. Co-culturing therefore seems to decrease ICAM-1 mRNA expression in HUVEC and MM6, by a mechanism that remains unknown.

MM6 cells appear to be necessary for the activation of HUVEC by plasma from preeclamptic women. Although the exact role of MM6 cells awaits further investigation, we suggest that plasma factors present in plasma of preeclamptic women activate MM6 cells and these in turn activate HUVEC.

MM6 cells in co-culture with HUVEC become activated by plasma from preeclamptic women, i.e. they show increased expression of ICAM-1 expression. Moreover, incubation with plasma from preeclamptic women also induced increased ROS production in co-cultured MM6 cells compared to incubation with plasma from nonpregnant women. To test whether extracellular ROS produced by MM6 (or by HUVEC themselves) in co-culture could activate HUVEC, we added SOD and catalase to the cultures. Interestingly, rather than the expected decrease in HUVEC ICAM-1 expression, we observed a significant increase in HUVEC ICAM-1 expression after incubation with SOD and catalase. This suggests that the extracellular ROS, produced by either MM6 cells or HUVEC, may not be involved in upregulating HUVEC ICAM-1 expression in the present experiments. Direct physical contact between MM6 and HUVEC or the production of cytokines by MM6 following plasma stimulation is alternative mechanisms by which MM6 cells could activate HUVEC. Further experiments are underway to test the role of these alternative mechanisms. The unexpected increase in HUVEC ICAM-1 expression following incubation with SOD and catalase may be explained by the fact that ROS increase the shedding of ICAM-1 from endothelial cells (Essick et al., 2008). Therefore, by inhibit-