Plasma From Women With Preeclampsia Inhibits Trophoblast Invasion

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To assess whether plasma from women with preeclampsia altered trophoblast invasion, SGHPL-4 extravillous trophoblasts were treated with pooled plasma from women with preeclampsia (PE-P; 10%) or with plasma from healthy pregnant controls (C-P). PE-P significantly inhibited SGHPL-4 invasion through Matrigel-coated transwells ($P < .01$), reduced mitochondrial dehydrogenase activity ($P < .01$), and increased apoptosis ($P < .05$); however, invading cells were no more susceptible to PE-P-induced apoptosis than their static counterparts. C-P did not alter rates of invasion, proliferation, or apoptosis. The bioactivity of PE-P was retained after removal of the 6 most abundant plasma proteins using an immunodepletion column ($P < .05$). Fractionation of PE-P demonstrated that the reduction in invasion was predominantly mediated by factors >100 kd in size. The authors conclude that plasma from women with preeclampsia contains multiple factors that inhibit invasion. These factors do not specifically target invading cells, but instead may reduce the number of cells available to invade.

KEY WORDS: Apoptosis, plasma, preeclampsia, proliferation, trophoblast invasion.

INTRODUCTION

The multisystem disorder of preeclampsia is a leading cause of maternal and perinatal morbidity and mortality, which affects up to 5% of pregnancies. The etiology of preeclampsia is still unknown despite its negative impact on both mother and fetus. Clinical outcomes include small-for-gestational-age babies, preterm delivery, and maternal or neonatal deaths. In addition, there is strong evidence linking the intrauterine environment to health in adulthood, that is, that there are “fetal origins of adult disease.”¹ Thus, the small babies resulting from pregnancies affected by preeclampsia have an increased risk of hypertension, heart disease, and diabetes in adult life. Women who have

had preeclampsia also have an increased risk of developing heart disease.

In the first 20 weeks of normal pregnancy, extravillous cytotrophoblasts Extravillous cytotrophoblast (EVT) detach from anchoring placental villi and invade the uterine wall or migrate through the lumen of the uterine spiral arteries as far as the first third of the myometrium. Trophoblast invasion initiates arterial remodelling, during which endothelial and smooth muscle cells are replaced by trophoblasts embedded in an amorphous fibrinoid matrix.²⁻⁴ This results in the formation of dilated, high-flow, low-resistance arteries that lack vasomotor control.⁵ In contrast, pregnancies complicated with preeclampsia are characterized by shallow trophoblast invasion, a decreased population of invasive trophoblast, and narrow bore arteries retaining muscular walls, reflecting an inherent failure of the trophoblast to elicit arterial transformation.⁶⁻⁸ There is accumulating evidence to suggest that the persistence of unmodified spiral arteries in the second and third trimester results in an impaired blood flow to the intervillous space, placental ischemia, and release of soluble factors from the placenta, which cause a systemic alteration of maternal vascular endothelial function and modify the behavior of resistance arteries.^{9,10} Thus, the

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failure of invasive trophoblast to mediate effective vascular transformation in the first trimester leads to the pathogenesis of preeclampsia later in gestation. We therefore assessed the effect of plasma from women with preeclampsia on trophoblast invasion *in vitro*, with the dual aims of elucidating its pathogenic effect on placental cells, and establishing an assay that may be useful in future studies of high-risk women. Plasma taken from women prior to the clinical onset of preeclampsia can alter endothelial cell function,¹¹ thus it is possible that the bioactivity identifiable in symptomatic women is also present before the onset of disease and may play a causative role.

MATERIALS AND METHODS

Reagents

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Harris hematoxylin, etoposide, Sigma Aldrich (Poole, UK); tissue culture medium and antibiotics, Cambrex (Wokingham, UK); fetal bovine serum, Alexa Fluor 568 goat anti-mouse immunoglobulin G (IgG), Invitrogen Ltd (Paisley, UK); Matrigel, BD Discovery Labware (Bedford, MA); Transwell tissue culture inserts with 8 μ m pores, Nunc (distributed by Fisher Scientific, Loughborough, UK); Vivaspin 500 spin columns, Sartorius, (Stockport, UK); *in situ* cell death detection kit (TUNEL), Roche (Lewes, UK); mouse anti-human Ki67 monoclonal antibody, rabbit anti-mouse fluorescein isothiocyanate (FITC) conjugated polyclonal antibody, DakoCytomation, (Denmark); Vectashield containing propidium iodide or DAPI, Vector Laboratories Inc, (Burlingame, CA); annexin V-FITC Apoptosis Detection Kit 1, BD Biosciences (Oxford, UK); multiple affinity removal column, Agilent (Wokingham, UK).

Study Group

Blood samples were taken from women at Ninewells Hospital, Dundee and at St Mary's Hospital, Manchester, at diagnosis of preeclampsia, and from normotensive control participants who were matched for gestation and parity. Women with preexisting medical conditions were not recruited and preeclampsia was diagnosed using standard definitions.¹² Patients were recruited into the study and their samples handled as described previously.¹⁰ Written informed consent was given for all plasma samples that were obtained and Local Research Ethics Committee approval was in place.

Plasma Samples

Blood samples were collected in precooled EDTA vials and centrifuged for 15 minutes at 1500 rpm at 4°C. The plasma was removed and stored at -80°C until required. Individual plasma samples were defrosted on ice and divided into 75 μ L aliquots; aliquots from multiple women were combined (Cohort 1, *n* = 12; Cohort 2, *n* = 23) to create pooled plasma samples from women with preeclampsia and from healthy pregnant controls. Aliquots of pooled plasma were stored at -80°C, defrosted on ice, and were maintained at 4°C during preparation of experiments. Pooled plasma from Cohort 2 was used for the experiments shown in Figure 5; pooled plasma from Cohort 1 was used for the remaining experiments.

Plasma Fractionation

Plasma was separated using Vivaspin columns with molecular weight cutoffs of 5, 10, 30, 50, and 100 kd. The Vivaspin membranes were prewashed with 200 μ L of phosphate buffered saline (PBS; 5 g/L NaCl, 0.25 g/L KCl, 1.44 g/L Na₂HPO₄, 0.24 g/L KH₂PO₄) and centrifuged at 11,500 g at 4°C for 10 minutes. The PBS was removed and 250 μ L of the pooled plasma from women with preeclampsia was loaded onto each column insert. The columns were centrifuged at 11,500g at 4°C for 30 minutes. Once finished, an additional 100 μ L of plasma was added to the column and the process was repeated. The columns were spun 3 times in total and on completion of the last cycle, both the eluent and retained fractions were recovered, made up to their original volume (250 μ L) with PBS, and stored at -80°C until use.

Multiple Affinity Removal System

A multiple affinity removal column was used to simultaneously remove the 6 highest abundance proteins from plasma (albumin, IgG, antitrypsin, immunoglobulin A, transferrin, and haptoglobin). The column was run, according to manufacturer's instructions, as previously described.¹⁰

Cell Culture

SGHPL-4 cells are derived from primary first trimester extravillous trophoblasts transfected with the early region of SV40; characterization has confirmed an extravillous trophoblast-like phenotype.^{13,14} SGHPL-4 cells were

cultured in Hams F10 media supplemented with glutamine (2 mmol/L), penicillin (100 IU/mL), streptomycin (100 µg/mL), and 10% (v/v) fetal bovine serum.

Transwell Invasion Assay

SGHPL-4 were plated into 10 mm transwell inserts with 8 µm pores (Nunc, UK), which had been precoated with a thin layer of Matrigel (BD Biosciences, UK) and placed into a 24-well plate. After the cells had adhered, the culture medium was removed and replaced with fresh medium containing 10% fetal bovine serum (FBS), with the addition of either 10% (v/v) PBS, 10% (v/v) pooled plasma from healthy pregnant controls (C-P), or 10% (v/v) pooled plasma from women diagnosed with preeclampsia (PE-P). Concurrently, the same number of cells were plated onto matching cover slips, which were cultured in parallel with PBS, C-P or PE-P. The cells were incubated at 37°C for 72 hours, after which time the transwells were washed with PBS and the cells fixed in 4% (w/v) paraformaldehyde in PBS for 15 minutes. The transwells were then removed from the plates and the upper side of each insert was wiped with a cotton bud to remove the cells. The cells on the lower side of the inserts and on the cover slips were stained with Harris' hematoxylin for 15 minutes and the number of cells per field was counted using a $\times 20$ objective. The number of invading cells (those present on the lower side of the transwell filter) were expressed as a percentage of the total number of cells at the end of the experiment (those present on the corresponding cover slip).

Mitochondrial Dehydrogenase (MTT) Assay

The mitochondrial dehydrogenase assay is widely used as a measure of cell viability, although it does not discriminate between apoptotic and necrotic cell death. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is reduced to formazan by mitochondrial dehydrogenases in healthy cells. The activity of this enzyme decreases as cells die, thus measuring formazan formation provides an estimate of cell viability. SGHPL-4 were seeded in 96-well plates and allowed to adhere. The culture medium was removed and replaced with fresh medium containing 10% FBS, with the addition of either 10% (v/v) PBS, 10% (v/v) C-P, or 10% (v/v) PE-P. After 24 hours, the culture medium was removed and replaced with medium containing 0.05% (w/v) MTT in PBS.

SGHPL-4 was incubated for a further 3 hours, the culture medium was removed and DMSO (100 µL/well) was added to the cells to solubilize the formazan crystals generated. After 1 to 2 minutes on a shaker, the absorbance of each well at 550 nm was measured using a plate reader.

Ki67 Proliferation Assay

The nuclear antigen Ki67 is a marker of proliferation that is only expressed in cycling cells; hence SGHPL-4 cells were immunostained with an antibody against Ki67 to determine the percentage of proliferating cells following treatment with the plasma samples. Briefly, SGHPL-4 were plated onto glass cover slips and allowed to adhere. The culture medium was then removed and replaced with fresh medium containing 10% FBS, with the addition of either 10% (v/v) PBS, 10% (v/v) C-P, or 10% (v/v) PE-P. After 24 hours, the culture medium was removed and the cells were fixed in 4% (w/v) paraformaldehyde dissolved in PBS for 15 minutes. After a brief wash with PBS, the cells were permeabilized following incubation with 0.1% (v/v) Triton-X100 dissolved in PBS solution for 8 minutes. Cells were then incubated with a mouse anti-human Ki67 primary antibody (1:50 dilution) for 1 hour at room temperature. Cells were washed 3 times in PBS and incubated with a rabbit anti-mouse secondary antibody conjugated to FITC (1:50 dilution) for 1 hour in the dark at room temperature. After 3 more PBS washes, cover slips were mounted on glass slides using Vectashield mounting medium containing propidium iodide. The percentage of proliferating cells was analyzed using a Zeiss fluorescence microscope.

Annexin V Apoptosis Assay

As cells undergo apoptosis, the cell membrane lipid phosphatidylserine is externalized. Annexin V binds specifically to phosphatidylserine, thus a commercially available Annexin V-FITC apoptosis detection kit was used to determine the percentage of apoptotic cells following treatment of the pooled plasma samples. Cells were grown on glass cover slips in culture medium containing 10% FBS and were treated with 10% (v/v) PBS, 10% (v/v) C-P, or 10% (v/v) PE-P. After 24 hours, the culture medium was removed and the cells were washed with PBS. The cover slips were then washed once in $1\times$ kit binding buffer and incubated in kit buffer containing annexin V solution (5% (v/v)) and propidium iodide (2.5 µg/mL) for 15 minutes, in the dark. Cells were

Table 1. Patient Demographic Data^a

	Preeclampsia, Cohort 1 (n = 12)	Normal Outcome, Cohort 1 (n = 12)	Preeclampsia, Cohort 2 (n = 23)	Normal Outcome, Cohort 2 (n = 23)
Max (systolic) BP (mm Hg)	150 ^b (140–178)	120 (110–147)	152 ^b (130–199)	124 (96–140)
Max (diastolic) BP (mm Hg)	100 ^b (94–110)	70 (60–91)	103 ^b (84–126)	78 (60–90)
Delivery gestation (weeks + days)	36 (33–39)	36 (28–40)	36 ^b (26–40)	40 (38–41)
Birth weight (g)	2300 ^b (1540–3250)	3380 (2620–4380)	2285 ^b (590–4780)	3480 (2670–4380)
individualized birth weight ratio (IBR) (centile)	13 ^b (0–53)	48 (11–99)	16 ^b (1–100)	38 (12–99)
Parity	0 (0–1)	0 (0–1)	0 (0–2)	0 (0–2)

^a Data represented as median (range).^b $P < .01$, preeclampsia versus normal outcome, Mann-Whitney U test.

IBR = individualized birth weight ratio

washed once in PBS and fixed using 4% (v/v) paraformaldehyde for 10 minutes. After a final wash in PBS, cover slips were mounted on glass slides using Vectashield mounting medium containing propidium iodide. The percentage of apoptotic cells was analyzed using a Zeiss fluorescence microscope.

Dual Staining of Transwell Filters

SGHPL-4 were seeded onto Matrigel-coated transwell inserts and incubated with either C-P or PE-P (10% (v/v)) for 24 hours in duplicate, in culture medium containing 10% FBS. After fixation with paraformaldehyde (4% (w/v) in PBS), cells were removed from either the upper or the lower side of each insert with a cotton bud. The filter was removed using a scalpel, placed on a microscope slide with the cells facing upward and dual-stained for markers of proliferation and apoptosis. Annexin V was not suitable for use in the dual staining protocol so TUNEL was used instead. Briefly, cells were permeabilized with Triton-X100 solution (0.1% (v/v) in PBS) for 8 minutes, washed in PBS, and incubated with TUNEL reagent in a humidified chamber for 1 hour at 37°C. The TUNEL reagent was prepared according to the manufacturer's instructions, although the enzyme was diluted 1:5 with PBS to reduce background fluorescence. The filters were washed in PBS, incubated with an anti-Ki67 primary antibody (1:50 dilution; 1 hour) and washed again. A goat anti-mouse Alexa Fluor 568 secondary antibody (1:200) was applied to the filters for 1 hour. Filters were then washed in PBS, mounted using Vectashield containing 4',6-diamidino-2-phenylindole (DAPI), and imaged using a Zeiss fluorescence microscope.

Statistics

Statistical analyses were performed using GraphPad Prism software. Nonparametric data is represented by medians and interquartile ranges and was analyzed with Friedman or Kruskal-Wallis tests with appropriate post hoc analysis. Significance was taken as $P < .05$. Data are from ≥ 3 independent experiments.

RESULTS

Patient characteristics are presented in Table 1. Women diagnosed with preeclampsia exhibited significantly higher systolic and diastolic blood pressure ($\star P < .01$; by definition) and delivered lower birth weight babies ($\star P < .01$). Control plasma samples were obtained from women with uncomplicated pregnancies and were matched for gestation and parity.

Trophoblast Invasion

To examine the effect of the pooled plasma samples on trophoblast invasion, SGHPL-4 cells cultured on Matrigel-coated transwells were incubated with PBS (10% (v/v)), pooled plasma from healthy control women (C-P), or pooled plasma from women with pre-eclampsia (PE-P) for 72 hours. PE-P significantly inhibited trophoblast invasion ($P < .01$), whereas C-P had no effect compared to PBS treated controls (Figure 1).

Trophoblast Viability

To determine whether the observed decrease in trophoblast invasion was the result of a decrease in viability, an MTT assay was performed. PE-P significantly reduced the

Figure 1. Plasma from women with preeclampsia inhibits the invasion of SGHPL-4. SGHPL-4 were incubated with phosphate buffered saline (PBS; 10% (v/v)), pooled plasma from healthy pregnant women (C-P; 10% (v/v)), or pooled plasma from women with preeclampsia (PE-P; 10% (v/v)). The percentage of the total cell population that was present on the lower side of the transwell inserts after 72 hours is shown. Median ($n = 3$); * $P < .05$, Dunn's comparisons of medians between C-P and PE-P.

mitochondrial dehydrogenase activity of SGHPL-4 after 24 hours ($P < .01$; Figure 2), while C-P was without effect, compared to PBS-treated controls. This suggested that factors present within PE-P either decreased trophoblast proliferation or increased trophoblast cell death. To investigate further, parallel cultures of SGHPL-4 were treated with C-P or PE-P for 24 hours and the rates of proliferation and apoptosis were analyzed. PE-P significantly increased phosphatidylserine externalization ($P < .05$), a marker of apoptosis (Figure 3), but had no effect on SGHPL-4 proliferation (Figure 4). C-P had no effect on trophoblast proliferation or apoptosis, compared to PBS-treated controls.

To investigate whether the observed decrease in invasion was directly due to PE-P-induced apoptosis of invading cells, proliferation and apoptosis were quantified *in situ*, on the upper and lower surfaces of the transwell inserts. Annexin V was not suitable for use in the dual-staining protocol so TUNEL was used as a marker of apoptosis. Again, PE-P significantly inhibited trophoblast invasion (data not shown). SGHPL-4 on each side of the inserts exhibited similar levels of Ki67 expression and TUNEL (Figure 5), hence PE-P did not reduce invasion by increasing rates of apoptosis in invading cells. A decrease in the basal rate of proliferation was observed when SGHPL-4 were cultured on Matrigel-coated transwells compared to uncoated glass cover slips (Figure 4), possibly reflecting the difference in matrix substrate on which the cells were

Figure 2. Plasma from women with preeclampsia decreases the mitochondrial dehydrogenase activity of SGHPL-4. SGHPL-4 were incubated with phosphate buffered saline (PBS; 10% (v/v)), pooled plasma from healthy pregnant women (C-P; 10% (v/v)), or pooled plasma from women with preeclampsia (PE-P; 10% (v/v)). Mitochondrial dehydrogenase activity of SGHPL-4 at 24 hours is expressed as percentage of control cell activity. Median ($n = 3$); * $P < .05$, Dunn's comparisons of medians test between C-P and PE-P.

cultured. PE-P treatment of SGHPL-4 cultured on transwells reduced the percentage of Ki67-positive cells on both sides of the filter, compared to cells treated with C-P; however, this decrease was not statistically significant (Figure 5).

Depletion of Highly Abundant Plasma Proteins

Plasma was depleted of the 6 most abundant plasma proteins, (albumin, IgG, antitrypsin, immunoglobulin A, transferrin, and haptoglobin) using a multiaffinity immunodepletion column. Future studies would aim to elucidate which plasma constituents were responsible for the detrimental effects on trophoblast invasion and viability, and depletion of highly abundant proteins is crucial to the detection of lower abundant species. Protein depletion did not reverse the inhibitory effect of PE-P on trophoblast invasion, indicating that the factors responsible are not strongly associated with these proteins ($P < .05$; Figure 6). However, a reduction in the basal rate of SGHPL-4 invasion was observed when cells were treated with protein-depleted C-P or PE-P, compared to that observed with whole plasma (Figure 1). Despite this, the magnitude of the inhibitory effect of PE-P was slightly increased. It is therefore possible that protein depletion removed one or more proinvasive or promigratory factors from the plasma.

Figure 6. Protein-depleted plasma from women with preeclampsia inhibits SGHPL-4 invasion. SGHPL-4 were incubated with protein-depleted, pooled plasma from healthy pregnant women (C-P-P; 10% (v/v)) or protein-depleted, pooled plasma from women with preeclampsia (PE-P-P; 10% (v/v)). The percentage of the total cell population that was present on the lower side of the transwell inserts after 72 hours is shown. Median ($n = 3$); $*P < .05$, Wilcoxon matched pairs test.

feedback whereby factors in the plasma of women with preeclampsia effect a decrease in trophoblast mitochondrial dehydrogenase activity, reduce invasion through a Matrigel-coated filter barrier, and increase apoptosis. Histopathological studies of the placental bed have shown that rates of apoptosis in interstitially migrating trophoblast are not elevated in preeclampsia;¹⁶ however, in the current study, both invading and nonmotile extravillous-like trophoblasts exhibited elevated apoptosis, suggesting that in vivo, precursor cells in placental villi, perhaps in cell columns, may be the targets of this pathogenic mechanism. At term, apoptosis is elevated both in villous tissue from preeclamptic placentas¹⁷ and in cytotrophoblasts isolated from them.¹⁸

A recent study demonstrated that plasma from women with preeclampsia stimulated transendothelial migration of neutrophils, a phenomenon that was attributed to enhanced production of interleukin 8 by the endothelial cell line employed.¹⁹ Other studies have shown effects of preeclamptic plasma on choriocarcinoma cell behavior, and candidate gene products including MMP-2 have been identified.²⁰ Furthermore, serum from women with preeclampsia has been shown to enhance the sensitivity of the HTR8 trophoblast cell line to Fas-induced apoptosis.²¹ In vivo, trophoblast invasion and vascular transformation are complete by 20 weeks,⁴ thus the extravillous-like cell line SGHPL4 was chosen for the current study, in preference to the

Figure 7. Effect of different molecular weight plasma fractions from women with preeclampsia on SGHPL-4 invasion. Plasma from women with preeclampsia was separated by ultrafiltration into (A) >5 , >10 , >30 , >50 , and >100 kd fractions and (B) <5 , <10 , <30 , <50 , and <100 kd fractions. SGHPL-4 cells were incubated with each fraction or with whole PE plasma (10% v/v; PE-P), normal plasma (10% v/v; C-P), or phosphate buffered saline (PBS; 10% v/v) for 24 hours. The percentage of the total cell population that was present on the lower side of the transwell inserts after 72 hours is shown. Median (A; $n = 3$), (B; $n = 5/6$).

choriocarcinoma or transformed cells that have been used previously, based on their similarity to first trimester invasive trophoblast.²²

Pooled plasma from women with preeclampsia retained the ability to inhibit invasion after removal of 6 highly abundant proteins that account for $>85\%$ of the plasma protein load; however, the basal rate of invasion was reduced. This implies that one or more factors that promote invasion may have been removed from the plasma during the depletion process. Separation on the basis of molecular mass demonstrated an inhibitory effect in fractions >100 kd. The decrease in invasion noted between cells treated with the <30 and <50 kd fractions indicates that molecules within this range may possess anti-invasive effects. Taken together, these data confirm the presence of multiple bioactive factors within the

plasma of women with preeclampsia, corroborating previous findings.^{10,11,23} Elucidation of the net biological effect of multiple changes in factors that regulate survival, migration, differentiation, and cell death will require extensive further study.

High uterine artery resistance in the first trimester of pregnancy is indicative of impaired arterial remodelling,²⁴ and is a risk factor for developing preeclampsia.²⁵ Indeed, serum collected from women with first trimester uterine artery Doppler resistance indices above the 95th centile inhibited the formation of trophoblast cell processes in a three-dimension (3D) fibrin gel.²³ However, first trimester extravillous trophoblasts isolated from the placenta of women with high uterine artery resistance at the time of pregnancy termination are more sensitive to apoptotic stimuli than trophoblasts obtained from women with a normal uterine artery resistance,²⁶ suggesting either a cell-intrinsic component in the pathogenic pathway or a sensitization process that persists *ex vivo*.

The eventual aim of this work is to identify effective predictors of preeclampsia and/or potential therapeutic targets to enhance trophoblast invasion prior to onset of the disease. Thus, a key issue is whether blood collected in first or second trimester in advance of the development of symptoms contains factors that inhibit invasion, potentially accounting for the incomplete vascular transformation that is characteristic of the disease. A second related point is whether the same factors observed to affect cell behavior in preeclamptic plasma are present in active concentrations at presymptomatic stages. Experiments using first trimester EVT treated with plasma obtained in the first trimester of pregnancy from women who go on to develop preeclampsia will answer these questions. It will also be necessary to address whether the observed cytotoxic effects of PE plasma are specific to invasive trophoblast or whether other lineages of trophoblast, vascular endothelial cells, or unrelated cell types respond in a similar manner. These studies will ascertain whether PE plasma reduces trophoblast differentiation and EVT egress from cell columns or whether it limits invasion via generalized cytotoxic effect. Current work in progress combines bioassays using SGHPL-4, as described herein, with sophisticated analytical technologies to separate, recombine, and identify protein or lipid candidates.

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