

Impaired vascular permeability regulation due to the VEGF_{165b} splice variant in pre-eclampsia

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Objective. Pre-eclampsia is diagnosed by hypertension and proteinuria, likely due to endothelial dysfunction, resulting in symptoms including oedema, inflammation and altered metabolism. Vascular Endothelial Growth Factor-A (VEGF) is detected at higher concentrations in plasma from patients with pre-eclampsia than normotensive pregnant patients when determined by radioimmunoassay. This study tested the hypothesis that circulating VEGF in pre-eclamptic plasma is biologically active *in vivo*, and aimed to identify specific isoforms responsible for this activity. Design. Plasma from pre-eclamptic (n=17) and normotensive (n=10) pregnant women was perfused into *Rana* mesenteric microvessels, and the subsequent change in microvascular permeability measured using a single vessel perfusion-microocclusion technique. Results. Pre-eclamptic but not normotensive plasma resulted in a 5.25 ± 0.8 fold acute increase in vascular permeability (p=0.0003). This increase could be blocked by incubation of plasma with bevacizumab, an antibody to VEGF (n=7, p=0.012), and by VEGF receptor inhibition, by SU5416 at doses specific to VEGF receptor-1 (VEGFR1), but not by the VEGF receptor-2 inhibitor, ZM323881. Although VEGF_{165b} levels were not significantly altered in the PET samples, the increase in permeability was also inhibited by incubation of pre-eclamptic plasma with an inhibitory monoclonal antibody specific for VEGF_{165b} (n=6, p<0.01), or by addition of Placental Growth Factor (PlGF-1, n=3, p<0.001). PlGF was detected at lower concentrations in pre-eclamptic plasma than in normotensive plasma. Conclusions. These findings suggest that circulating VEGF levels in pre-eclampsia are biologically active, due to loss of repression of VEGFR1 signalling by PlGF-1, and VEGF_{165b} may be involved in the increased vascular permeability of pre-eclampsia.

Keywords. Pre-eclampsia, VEGF, microvascular permeability, hydraulic conductivity.

Introduction

Pre-eclampsia complicates around 3-5% of all pregnancies¹, has the cardinal features of pregnancy induced hypertension and proteinuria, and is associated with endothelial dysfunction resulting in wide spread maternal symptoms, including oedema. It is thus widely accepted to be an endothelial cell disorder, with activation of the systemic inflammatory network. However, the specific factors causing this microvascular dysfunction are still unknown². The vasculature of a pregnant woman with pre-eclampsia is vasoconstricted (from smooth muscle contraction)^{3,4}, and is in a state of greater increased vascular permeability throughout the body than normotensive pregnant women^{5,6}. This pro-permeable state was first recognised in the 1980s, when the accelerated disappearance of Evans Blue dye was observed from the plasma of pre-eclamptic compared to normotensive women⁷. Pre-eclamptic women also have a lower plasma volume than healthy pregnant women⁸. Analysis of transcapillary fluid balance in pre-eclamptic women suggests that in severe pre-eclampsia there is increased filtration of plasma proteins from the capillary lumen to subcutaneous tissue, because in severe pre-eclampsia high interstitial, but low plasma colloid osmotic pressures are observed⁵. This explains the clinical findings of oedema and proteinuria. Pre-eclampsia resolves when the placenta is delivered, which, combined with the systemic nature of the condition has led to the hypothesis that a circulating factor, possibly sourced from the placenta, was responsible for the clinical symptoms of pre-eclampsia. There have been many studies investigating the role of circulating factors in pre-eclampsia, and a number of molecules including VEGF⁹, soluble VEGFR1¹⁰, endoglin¹¹, and placental protein 13¹² have been shown to be altered in pre-eclampsia, and the VEGF family members are of particular interest as they are pro-permeability, pro-angiogenic vasodilators. All three of these properties are associated with pre-eclampsia. There are five members of the VEGF family in humans, VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF, the most studied of which is

VEGF-A. However, while VEGF-A is known to increase permeability¹³, it is a vasodilator¹⁴, and could not account for increased peripheral resistance and hence hypertension. Moreover, plasma VEGF levels have been measured by two different techniques to give two different outcomes. Measurement by sandwich ELISA shows that VEGF levels in plasma fall with pre-eclampsia¹⁵. In contrast measurement by competitive enzyme immunoassay, or radio-immunoassay show that VEGF levels rise with pre-eclampsia^{16,17}. As sVEGFR1 levels also rise with pre-eclampsia it has been suggested that free, active VEGF levels are those measured by ELISA, whereas bound, inactive and active VEGF levels (i.e. total VEGF) are measured by cEIA and RIA.

A number of studies in endothelial cells in culture and isolated vessels *ex vivo* have shown that pre-eclamptic plasma has biological activity, including production of superoxide¹⁸, inflammatory mediators^{19,20,21}, increased monolayer permeability through protein kinase C activation²², effects on membrane protein expression^{23,24} and vasodilatation²⁵. In 2004, direct evidence that pre-eclamptic plasma contained a macromolecule that was biologically active *in vivo* was published. Experiments were performed in which frog microvessels were perfused with either dialysed plasma from non-pregnant, pre-eclamptic or normotensive pregnant women²⁶. An acute, transient increase in vessel permeability was observed during perfusion with pre-eclamptic, but not plasma from women with uncomplicated pregnancies, allowing the conclusion that an (as yet) unidentified macromolecule must be present in the plasma of pre-eclamptic women, which is responsible for this rise in permeability in this experimental model. One potential candidate for this is vascular endothelial growth factor (VEGF), but only if it was present in an active form in pre-eclamptic, but not plasma from women with uncomplicated pregnancies. Recently a novel VEGF isoform, VEGF_{165b} was discovered^{27,28} which is formed by splicing at the distal rather than proximal splice site of exon 8 of the VEGF gene²⁹, and is only partially detected by the commercial

VEGF ELISAs³⁹. VEGF_{165b} has also been shown to transiently increase vessel permeability³⁰. As it has not been determined whether VEGF in pre-eclamptic plasma was biologically active, we investigated whether the VEGF family was responsible for the increased microvascular permeability induced by pre-eclamptic plasma in this experimental model, and which isoforms and receptors were involved.

Methods

Ethical approval was obtained from the local ethics committee (United Bristol NHS Trust, Pretoria Academic Hospital) and conforms to the Declaration of Helsinki. Pregnant patients with and without pre-eclampsia were recruited after obtaining consent, from St Michael's Hospital, Bristol, and Pretoria Academic Hospital, South Africa. Patients were diagnosed with severe pre-eclampsia based on the Royal College of Obstetricians and Gynaecologists Green-top Guidelines for the Management of Severe Pre-eclampsia, which requires the presence of diastolic blood pressure of ≥ 110 mmHg or systolic blood pressure of ≥ 170 mmHg, together with ≥ 1 g/litre/24hours proteinuria. Pre-eclampsia was diagnosed in the presence of pregnancy induced hypertension $\geq 140/90$ mmHg and ≥ 0.3 g/litre/24hours proteinuria⁴⁵. Following consent, 20ml blood was taken into lithium heparin tubes and immediately centrifuged at 4000rpm at 5°C. Plasma samples were taken before the onset of labour, and stored at -80°C. After thawing, plasma was dialysed against frog Ringer's solution in 14,000 Dalton dialysis tubing to normalise the plasma for serum sodium concentration (in frogs this is approximately 120mmol/l), and filtered through a 0.2µm sterile filter. The oncotic pressure of the plasma sample was measured using a Pantos oncometer. Table S1 shows the clinical characteristics of the study population.

Permeability Studies

The Landis Michel Micro-occlusion technique was used to measure microvascular permeability of intact vessels *in vivo*, in the presence of normotensive and pre-eclamptic plasma in accordance with UK Home Office regulations. This technique, which was first described in 1974^{31, 32}, has been widely evaluated in the literature³³. It uses Starling's equation, which states that the fluid movement due to filtration across the wall of a capillary is dependent on the balance between the hydrostatic pressure and the oncotic pressure gradients across the capillary³⁴. The technique involves the cannulation of a microscopic vessel from the mesen-

tery of an anaesthetised frog, and perfusing the vessel with plasma under investigation (which contains a few erythrocytes to act as flow markers) at a known hydrostatic pressure. By occluding the cannulated vessel several hundred micrometres downstream, any further flow of red blood cell markers towards the occlusion site occurs as a result of fluid filtration across the capillary wall. This method therefore measures the barrier properties of the capillary wall without direct contributions from circulating haemodynamic forces such as increased blood flow, pressure or surface area, a problem with studies involving dye extravasation of for instance, Evans' blue³⁵.

Experiment Preparation

Male *Rana pipiens* frogs (weight 25-30g, purchased from Charles D. Sullivan, Nashville Tennessee) were anaesthetised by immersion in 1mg/ml MS222 (3-aminobenzoic acid ethyl ester) in water, and anaesthesia maintained by superfusion with 0.25mg/ml MS222 in frog Ringer's solution (NaCl 6.49g/l, CaCl₂·2H₂O 0.15g/l, KCl 0.269g/l, MgCl₂·6H₂O 0.2g/l, glucose 0.99g/l, NaHCO₃ 0.015g/l, HEPES salt 0.61g/l, HEPES acid 0.627g/l). The frog is an ideal model for *in vivo* vascular permeability measurement because their vessels do not express the Gal α 1:3Gal antigen which is responsible for xenograft tissue rejection due to preformed antibodies to Gal α 1:3Gal in mammals³⁶. The frog was laid supine on a Perpex mount, a 10 mm incision made in its lower left anterior abdominal wall, and the distal ileum gently floated out using a cotton wool swab and draped over a transparent Perpex pillar. The upper surface of the mesentery was continuously superfused with frog Ringer's solution (pH 7.40 \pm 0.05) and all experiments were performed at room temperature (20-22°C). The mesentery was viewed using a microscope connected to a video camera, video-cassette recorder (Panasonic AG7350), microphone and timer for the simultaneous recording of experiments, which could be viewed and analysed at a later time.

Measurement of Hydraulic Conductivity (Lp)

Vessels were selected that were 15-35 μ m in diameter, over 700 μ m in length, with no side branching or leucocyte margination, and freely flowing. The vessel was cannulated with a glass micropipette ground to an open point 12-15 μ m in diameter filled with 4-6% human serum albumin in frog Ringer with washed erythrocytes as flow markers and connected to a micropipette holder. The albumin concentration was matched to the known oncotic pressure of the plasma samples to be tested. This system was attached to a bifurcated water manometer, set at 2 pressures (15 and 35 cmH₂O), and the vessel was then occluded for 5 seconds by a glass tube held in a micromanipulator, during which time the pressure was switched from 15 to 30 cmH₂O and back again. The occluding glass tube was then removed to allow the perfusate to flow freely through the microvessel for at least 5 seconds before another occlusion was made. Each experiment was run for a maximum of 5 minutes, and each vessel was used for only one experiment.

Calculation of L_p

The transcapillary fluid filtration rate (J_v/A) per unit area of vessel wall was calculated from the red blood cell (RBC) velocity (dl/dt) after vessel occlusion at known pressure, the vessel radius (r) and the distance (l) from the RBC to the occlusion site. These measurements were taken from the video-tape as the experiment was recorded.

$$J_v/A = (dl/dt) (r/(2l)) \quad \text{Equation 1}$$

The filtration rate was recorded at 2 pressures (15, 30 and back to 15 cmH₂O over a period of 5 seconds) and the Starling equation was used to calculate the L_p and reflection coefficient (σ):

$$J_v/A = L_p [(P_c - P_i) - \sigma (\pi_c - \pi_i)] \quad \text{Equation 2}$$

Where P_c is the capillary hydrostatic pressure, P_i the interstitial hydrostatic pressure, π_c the capillary oncotic pressure and π_i the interstitial oncotic pressure.

Because J_v/A is measured at 2 pressures the overall L_p is:

$$L_p = (J_{v2}/A - J_{v1}/A) / P_2 - P_1 \quad \text{Equation 3}$$

Where J_{v2}/A is the average filtration rate at time points t_1 (usually within the first 2 seconds, during the lower applied pressure) and t_3 (in the last 1-2 seconds of the occlusion, again at the lower pressure), and J_{v1}/A is the measurement at time point t_2 (usually 2-3 seconds after perfusion, during the higher applied pressure), and the average of t_1 and t_3 is t_2 . Therefore J_{v2}/A is an approximation of what it would have been at t_2 , so equation 3 would be valid when J_v/A is quickly changing.

Enzyme linked immunoassay for VEGF₁₆₅b quantification

This ELISA has recently been described in detail³⁷. Anti-VEGF₁₆₅b antibody (MAB3045, clone 56/1, R&D systems) was coated onto an Immulon-2HB 96 well plate at 200µg/ml overnight. This antibody recognises an epitope within a 9 amino acid sequence at the C terminus of human VEGF₁₆₅b. The plate was washed with 1xPBS-Tween 0.05%, and then blocked for 12 hours with Superblock (Pierce 37515). Serial dilutions of recombinant VEGF₁₆₅b standards (R&D systems) diluted in 1xPBS/1%BSA, and plasma samples were then added to the wells in triplicate. Plates were incubated at room temperature with shaking for 2 hours and then washed as above. 50ng/ml of anti-human VEGF biotinylated affinity purified polyclonal antibody (BAF293, R&D systems) was added and incubated in foil for 2 hours as a detection reagent. After washing, 100µl HRP-streptavidin diluted 1 in 200 in 1xPBS was added for 20 minutes in foil, and then substrates A and B were added following washing. The colour change was stopped on addition of 1M H₂SO₄ (50µl/well) and plates were read at a wavelength of 450nm using a plate photospectrometer (Dynex Technologies). Revelation Quicklink 4.25 software was used to construct a standard curve from mean absorbance values of VEGF₁₆₅b standards, which enabled estimation of VEGF₁₆₅b concentration in plasma samples. The mean±SD coefficient of variation of repeated samples from the

same patient (inter-assay COV) was $19 \pm 11\%$, $N=10$. Neither sFlt-1, VEGF₁₆₅ or VEGF₁₂₁ interfere with this ELISA³⁹.

Statistics

Wilcoxon paired tests, Unpaired t tests and Mann Whitney U tests were used to compare data sets and a p value of < 0.05 was regarded as significant. Changes in permeability were expressed as fold increase in permeability from the average baseline Lp reading immediately prior to perfusion with the plasma under investigation, to the maximum reading obtained during the experiment. Results are expressed as mean \pm S.E.M.

Results

Plasma from women with pre-eclampsia increases microvascular permeability

Frog mesenteric vessels were perfused with dialysed plasma from 10 normotensive pregnant women in their third trimester for up to 10 minutes. During this perfusion there was no clear transient change in permeability, and there was no statistically significant increase in vessel permeability (mean peak \pm SEM: 1.95 ± 0.45 fold compared with HSA, Figure S1A, B).

Vessel perfusion with dialysed plasma from 17 women with pre-eclampsia resulted in a rapid transient increase in vascular permeability, which returned to baseline after a few minutes, similar to that previously described²⁶. The mean \pm SEM of the peak increase was 5.25 ± 0.8 fold greater than the baseline measurements, Figure S1C, Wilcoxon, $p=0.0003$). An example of a vessel's response to perfusion with pre-eclamptic plasma is shown in Figure S1D. This patient presented at 29 weeks gestation with severe pre-eclampsia and ultrasound evidence of fetal growth restriction secondary to pre-eclampsia.

Circulating VEGF_{165b} is found in the plasma of pregnant women

Circulating levels of VEGF_{165b} present in the plasma under investigation were quantified using the ELISA described above. VEGF_{165b} is highly expressed during normotensive ($4.6\text{ng/ml} \pm 2.3$) and pre-eclamptic ($5.9\text{ng/ml} \pm 2.7$) pregnancy (Figure S2A), which is consistent with previous observations^{38, 39}. However, there was no difference in plasma VEGF_{165b} levels between healthy pregnant women and pre-eclamptic patients, and no correlation existed between VEGF_{165b} concentration and fold change in microvascular permeability (Figure S2B).

The acute increase in microvascular permeability can be blocked by an antibody to VEGF

Plasma from 7 pre-eclamptic patients was perfused into 7 microvessels, resulting in a 6.1 ± 0.85 -fold increase in Lp. These experiments were repeated with plasma incubated with bevacizumab 25nmol/L prior to the experiment. Bevacizumab is a monoclonal antibody

that binds and inactivates all VEGF-A isoforms^{38, 39}. Perfusion of pre-eclamptic plasma with bevacizumab inhibited the rise in Lp previously seen, with the average increase in Lp being only 1.6-fold \pm 0.26 (Figure 1A, Mann Whitney U test, $p=0.0012$ compared with pre-eclamptic plasma alone, Figure 1B, an example experiment).

Inhibition of VEGF receptors prevents the pre-eclamptic rise in microvascular permeability

To determine whether the increase in permeability induced by pre-eclamptic plasma was dependent upon VEGF receptor (VEGFR) signalling, both VEGFRs in the vessel were inhibited (ZM323881 inhibits VEGFR-2 at 10nmol/L; SU5416 inhibits VEGFR-1 at 200nmol/L³⁰). Plasma from 7 patients with pre-eclampsia caused a 5.07 ± 0.67 rise in Lp. When these same plasma samples were perfused in the presence of both VEGF inhibitors, no subsequent significant acute increase in vascular permeability was seen (Figure 1C, Mann Whitney U test, $p=0.0041$; and an example experiment, figure 1D).

Specific inhibition of VEGFR-1 prevents the pre-eclamptic rise in microvascular permeability

Plasma samples from 10 women with pre-eclampsia were perfused into 10 microvessels, resulting in a transient increase in permeability of 6.2 ± 1.3 fold. To our surprise, when these experiments were repeated with 200nmol/L SU5416 in the plasma, a dose at which this inhibitor is specific for VEGFR-1, the ability of the plasma to increase vessel permeability was abolished (Figure 2A, 1.9 ± 0.3 fold, Mann Whitney U test, $p = 0.0015$; and an example experiment shown in figure 2B). Furthermore, when the experiment was repeated with 10 nmol/L ZM323881 ($n=8$), an inhibitor that specifically blocks VEGFR-2 only, the pre-eclamptic rise in permeability still occurred (Figure 2C, 4.9 ± 1 fold, with an example experiment in 2D). These results indicate that the mechanism of the VEGF dependent increase in permeability is VEGFR-1 dependent, but not VEGFR2 dependent. We have previously published results using this model that suggested that the permeability increase induced by

VEGF family members is VEGFR2 dependent, not VEGFR1 dependent, as VEGF-C (acts on VEGFR2 and 3) but not PIGF (acts on VEGFR1) increases permeability in this model³⁸.³⁹. However, we have also shown that the anti-angiogenic isoform of VEGF, VEGF_{165b} can transiently increase hydraulic conductivity, but not in the presence of 200nM SU5416^{38,39} implying that this effect of VEGF_{165b} is mediated by VEGFR1. We therefore sought to block VEGF_{165b} using a neutralising antibody specific for VEGF_{165b}.

The rise in microvascular permeability induced by pre-eclamptic plasma can be blocked, by specifically inhibiting VEGF_{165b} in maternal plasma

Incubation of pre-eclamptic plasma with 25nmol/L of a monoclonal antibody against the C terminus of VEGF_{165b} (R&D systems, MAB3045) prior to vessel perfusion significantly inhibited the Lp increase, with only a 1.3 ± 0.2 fold increase (Mann Whitney U test, $p=0.0022$, compared with pre-eclamptic plasma alone, $n=12$, Figure 3A, example shown in Figure 3B). To prove that IgG alone cannot inhibit the permeability rise we performed the experiments with pre-eclamptic plasma incubated with control IgG. No inhibition of the permeability increase occurred ($p=0.5$).

To confirm that the permeability enhancing effects of only VEGF_{165b} and not VEGF₁₆₅ are blocked by SU5416, the Landis Michel technique was performed in which aliquots of 5%HSA containing VEGF₁₆₅, VEGF_{165b}, VEGF₁₆₅ & SU5416 200nmol/L or VEGF_{165b} & SU5416 200nmol/L were perfused into microvessels in 25 separate experiments. Both VEGF₁₆₅ and VEGF_{165b} increased the Lp by 4.9 ± 1.2 and 3.5 ± 0.8 fold respectively. The increase in permeability was inhibited when perfused with VEGF_{165b} & SU5416 (1.6 ± 0.5 fold) consistent with previous experiments³⁰, but not with VEGF₁₆₅ & SU5416 (5.5 ± 2.1 fold, Figure S3). Equally to confirm that ZM323881 inhibited the permeability increase caused by VEGF₁₆₅ as previously described⁴¹ but not VEGF_{165b} vessels were perfused with ZM323881 and the response to VEGF isoforms measured. The response to VEGF_{165b} was

not affected (3.3 ± 0.3 fold), whereas the effect of VEGF₁₆₅ was inhibited (1.0 ± 0.09 fold compared with before VEGF treatment). The percent increase relative to that with VEGF alone is shown in figure S3. .

The rise in microvascular permeability induced by pre-eclamptic plasma can be blocked, by restoring PlGF-1 to maternal plasma

There was no significant difference in plasma VEGF_{165b} levels between pre-eclamptic patients and women with uncomplicated pregnancies (figure S2B). We therefore hypothesised that VEGF_{165b} is responsible for the biological activity of VEGF in pre-eclamptic plasma, not because VEGF_{165b} concentrations rise, but because a factor that blocks the VEGFR1-mediated activity of VEGF_{165b} in normotensive pregnancy is lost in pre-eclampsia. As VEGF_{165b} acts through VEGFR1 and PlGF also acts on VEGFR1 we measured PlGF levels. PlGF levels were substantially reduced in the plasma of patients with pre-eclampsia (55 ± 7 pg/ml vs 353 ± 60 pg/ml, $p < 0.0001$, Mann Whitney U test). To determine whether PlGF could inhibit the increase in plasma we incubated pre-eclamptic plasma with 10nmol/L of recombinant PlGF-1 (R&D systems, MAB3045) prior to vessel perfusion. This significantly inhibited the Lp increase, with only a 1.3 ± 0.2 fold increase (Mann Whitney U test, $p < 0.001$, $n=12$, Figure 3C, example shown in Figure 3D).

Discussion

We show here that the increased microvascular permeability in an *in vivo* experimental model induced by plasma from pre-eclamptic but not normotensive pregnant women is VEGF-A dependent, as it is inhibited both by the VEGF-A specific neutralising antibody, bevacizumab, and by inhibition of VEGFR phosphorylation. These results are consistent with the finding by Brockelsby et al that the inhibition of endothelial dependent relaxation induced by perfusion of mammalian vessels *ex vivo* with pre-eclamptic plasma is blocked by VEGF-A antibodies³.

However, we were surprised to find that it was the anti-angiogenic family of VEGF isoforms that was responsible for the biological activity of VEGF in pre-eclamptic plasma, and not through VEGFR-2 but through VEGFR-1⁴⁰, inhibition of which abolishes the previously observed pre-eclamptic-plasma-induced rise in permeability. VEGF_{165b} and VEGF₁₆₅ have similar binding kinetics to VEGFR1 and VEGFR2⁴⁶. These findings strongly suggest that the permeability increase in this model is mediated by circulating VEGF_{165b} in the plasma. VEGF_{165b} has been found to be expressed in many tissues as both protein and mRNA, at significant levels²⁹. Its function is still being determined, but it has now been shown to play roles in development of the reproductive system^{47, 48}, lactation⁴⁹, renal function⁵⁰, and prevention of ocular neovascularisation⁵¹. Its role in healthy pregnancy has yet to be defined, but there is no difference in VEGF_{165b} levels in healthy versus pre-eclamptic patients³⁷, precluding the concept that it is raised VEGF_{165b} levels that are responsible for the VEGF_{165b} induced increase in permeability induced by pre-eclamptic plasma in this model. Thus the most simple explanation for these apparent contradictory findings are that the VEGF_{165b} mediated permeability increase in this model is repressed in uncomplicated pregnancies, and this repression is lifted in pre-eclampsia. This would suggest the absence rather than the presence of a circulating factor in pre-eclampsia. Placental Growth Factor (PlGF-1), from

the VEGF family, shows reduced expression in pre-eclampsia⁴², albeit by ELISA. This may be interfered with by circulating sFlt-1 levels, although the circulating Flt1 levels in normal plasma (2-3ng/ml) are more than sufficient to bind PlGF (~600pg/ml) suggesting that the reduction in PlGF is not due to binding of sFlt1. However, irrespective of the reason for the reduction in bioavailable PlGF-1, it only interacts with VEGFR-1⁴³, and does not increase vascular permeability in the system used here⁴⁴. For this reason we tested the effect of additional PlGF-1 on the permeability increase induced by PET. Increasing circulating levels of PlGF-1 blocked the pre-eclampsia induced permeability increase.

These findings therefore raise the intriguing concept that the biological activity of pre-eclamptic plasma seen in this model of permeability measurement is due to a loss of bioavailable PlGF-1, resulting in loss of repression of the activity of VEGF_{165b}. Thus in pregnancy, when VEGF levels are raised (and not in non-pregnant situations when VEGF levels are not so high), PlGF-1 is required to prevent circulating VEGF_{165b} from exerting a biological effect. The clinical implications of these findings might be profound, but we should take care not to extrapolate the findings here to the clinical situation. A number of caveats need to be raised. We have measured the biological activity of circulating plasma taken ex vivo in an in vivo xenogeneic model of capillary barrier function. This model is not applicable to the human condition. Specifically it measures a relatively transient, physiologically unimportant (directly), consequence of circulating macromolecules in the amphibian. Thus it is clear that experiments are needed to determine to what extent the circulating effects of human plasma on endothelial cells in culture, ex vivo and in vivo are also regulated through PlGF-1 mediated loss of repression of the activity of the VEGF isoforms. However, monolayer permeability²², superoxide¹⁸, IL-6²⁰, and IL-8²¹ production, cadherin rearrangement²³, VCAM, P-selectin and E-selectin expression²⁴ and prostacyclin production¹⁹ have all been shown to be activated by pre-eclamptic plasma on human endothelial cells.

Moreover, pre-eclamptic plasma results in impaired vasodilatation of human arteries *ex vivo*²⁵. The extent to which these effects are mediated by loss of VEGF repression by PlGF-1 reduction needs to be determined. If these are also similarly affected, then it is possible that PlGF-1 replacement therapy might have some role to play in the management of pre-eclampsia.

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Conflict(s) of Interest/Disclosure(s)

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Ethics approval statement

Ethics approval was under NRES number 06/Q2006/54, Vascular Permeability in Pre-eclampsia, NRES, Central and South Bristol. 19/10/2006

Contribution to Authorship

VLB, DOB, PWS, SJH, CRN conceived and designed the experiments, VLB and AHS performed the experiments, VLB, AHS and DOB analyzed the data. VLB, BJ, TGO and PWS contributed clinical samples and information, VLB, SJH, DOB, PWS wrote the paper.

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Figure Legends

Table S1. Clinical characteristics of the study population

Figure S1. Pre-eclamptic plasma increases hydraulic conductivity of frog microvessels.

A. Frog mesenteric microvessels were cannulated and perfused with dialysed plasma from healthy pregnant women (n=10). No significant increase in hydraulic conductivity (L_p) was observed. B. Example of a single experiment: baseline microvascular permeability during perfusion with human serum albumin (HSA open symbols) followed by perfusion with normotensive plasma at time 0 seconds resulted in no significant change in vessel permeability. C. Perfusion of frog mesenteric microvessels with dialysed plasma from pre-eclamptic women (n=17, Wilcoxon test, $p=0.0003$). D. Example of a single experiment: perfusion with pre-eclamptic plasma at time 0 seconds resulted in an acute, transient increase in vessel permeability compared with HSA that occurred within 1 minute had returned to baseline by 3 minutes.

Figure S2. VEGF_{165b} in plasma. A. ELISA was used to quantify the concentrations of VEGF_{165b} both in the normotensive and pre-eclamptic plasma (n=27). There was no statistical significant difference (Unpaired t test, $p=0.73$). Values are expressed as mean \pm S.E.M. Bar shows medians. B. No correlation exists between VEGF_{165b} concentration and the change in hydraulic conductivity of the microvessel during perfusion with maternal plasma containing the VEGF_{165b}.

Figure S3. The effect of SU5416 and ZM323881 on the percentage fold increase of hydraulic activity (L_p) mediated by VEGF₁₆₅ and VEGF_{165b}.

. 1%HSA containing 1nmol/L VEGF₁₆₅, 1nmol/L VEGF_{165b}, 1nmol/L VEGF₁₆₅ & 200nmol/L SU5416, 1nmol/L VEGF_{165b} & 200nmol/L SU5416, 1nmol/L VEGF₁₆₅ & 10nmol/L ZM323881 or 1nmol/L VEGF_{165b} & 10nmol/L ZM323881 was perfused into frog mesenteric microvessels (n=16). SU5416 prevented the rise in permeability occurring during perfusion with VEGF_{165b} only. ZM323881 inhibited the VEGF₁₆₅ mediated acute rise in L_p (Mann Whitney U tests, $p<0.05$).

Figure 1. The permeability increase is VEGF dependent. A. Plasma from 7 women with pre-eclampsia was perfused into microvessels at known pressure. This resulted in an increase in L_p . Experiments were repeated in different vessels with pre-eclamptic plasma from the same 7 patients, which had been incubated for 1 hour prior to perfusion with 25nmol/L bevacizumab, which inhibited the rise in L_p (Mann Whitney U test, $p=0.0012$). B. Example

of two experiments. Perfusion of pre-eclamptic plasma increased Lp (closed circles). Pre-incubation with 25nmol/L bevacizumab (triangles) resulted in no change in permeability. **C.** Perfusion of plasma from 7 pre-eclamptic women into microvessels previously perfused with 200nmol/L SU5416 (selectively inhibits VEGF receptor-1) and 10nmol/L ZM323881 (selectively inhibits VEGF receptor-2) prevents the acute rise in vascular permeability observed during perfusion with pre-eclamptic plasma alone (Mann Whitney U test, $p=0.0041$). **D.** Example of a single experiment: perfusion of pre-eclamptic plasma after perfusion of the vessel with VEGFR-1 and VEGFR-2 inhibitors resulted in no significant change in vessel permeability.

Figure 2. PET plasma induced increased vascular permeability is blocked by VEGFR1 not VEGFR2 inhibition. **A.** Plasma from 10 women with pre-eclampsia was perfused into microvessels and an increase in Lp was observed. When a vessel was perfused with 200nmol/L SU5416 to inhibit VEGFR-1, subsequent perfusion of the same pre-eclamptic plasma resulted in a blunted response (Mann Whitney U test, $p = 0.0015$). **B.** Example of a single experiment. **C** Pre-eclamptic plasma ($n=8$) was perfused into vessels and permeability measured. The same plasma samples were then perfused into vessels that had been perfused with 10 nmol/L ZM323881 to inhibit VEGFR-2. No significant inhibition of the pre-eclamptic plasma-induced permeability increase was observed. (Unpaired t test, $p=0.57$, $n=8$) **D.** Example of a single experiment: the acute rise in vascular permeability seen in the absence of ZM323881 (closed circles) was still seen to occur (closed triangles).

Figure 3. VEGF_{165b}, and lack of PlGF is responsible for the increase in permeability. **A.** Incubation of pre-eclamptic plasma with 25nmol/L anti-VEGF_{165b} antibody prior to perfusion of capillaries with pre-eclamptic plasma does not result in the 5-fold increase in vascular permeability previously seen during perfusion with that pre-eclamptic plasma alone (Mann Whitney U test, $p=0.0022$, $n=12$). **B.** Example of a single experiment: perfusion of pre-eclamptic plasma with a monoclonal antibody against VEGF_{165b} (closed triangles) or pre-eclamptic plasma alone (closed circles). **C.** Incubation of pre-eclamptic plasma with 10nM PlGF-1 prior to perfusion of capillaries with pre-eclamptic plasma does not result in the increase in vascular permeability previously seen during perfusion with pre-eclamptic plasma alone **D.** 10nM PlGF-1 significantly inhibits the pre-eclamptic plasma mediated permeability increase (paired t test, $p<0.001$, $n=3$).

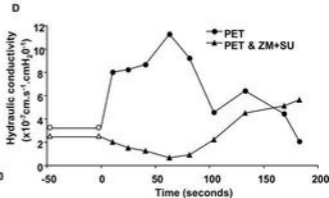
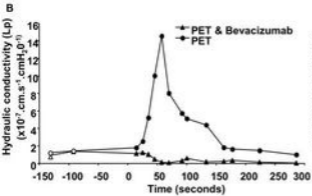
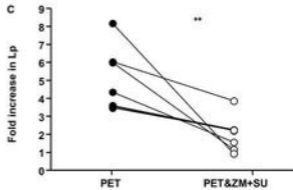
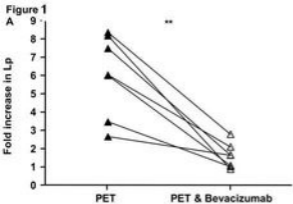


Figure 2

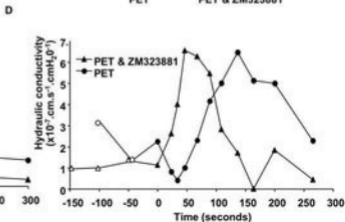
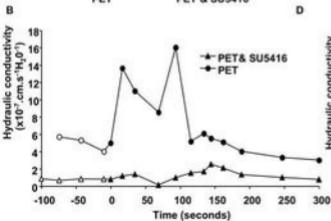
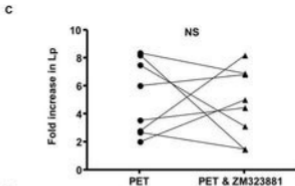
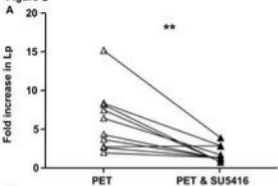


Figure 3

