An unexpected tail of VEGF and PlGF in pre-eclampsia

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Abstract
PET (pre-eclamptic toxaemia), characterized by pregnancy-related hypertension and proteinuria, due to widespread endothelial dysfunction, is a primary cause of maternal morbidity. Altered circulating factors, particularly the VEGF (vascular endothelial growth factor) family of proteins and their receptors, are thought to be key contributors to this disease. Plasma from patients with PET induces numerous cellular and physiological changes in endothelial cells, indicating the presence of a circulating imbalance of the normal plasma constituents. These have been narrowed down to macromolecules of the VEGF family of proteins and receptors. It has been shown that responses of endothelial cells in intact vessels to plasma from patients with pre-eclampsia is VEGF-dependent. It has recently been shown that this may be specific to the VEGF165b isoform, and blocked by addition of recombinant human PlGF (placental growth factor). Taken together with results that show that sVEGFR1 (soluble VEGF receptor 1) levels are insufficient to bind VEGF-A in human plasma from patients with pre-eclampsia, and that other circulating macromolecules bind, but do not inactivate, VEGF-A, this suggests that novel hypotheses involving altered bioavailability of VEGF isoforms resulting from reduced or bound PlGF, or increased sVEGFR1 increasing biological activity of circulating plasma, could be tested. This suggests that knowing how to alter the balance of VEGF family members could prevent endothelial activation, and potentially some symptoms, of pre-eclampsia.

Introduction
Pre-eclampsia [or PET (pre-eclamptic toxaemia)] occurs in 3–5% of first pregnancies and is characterized by widespread endothelial dysfunction [1], resulting in clinical vascular manifestations including hypertension, proteinuria, cerebral oedema and infarction, eclampsia (seizures), pulmonary oedema, liver haemorrhage, renal failure and coagulopathy. The clinical picture is resolved with removal of the placenta, suggesting a placental source for the systemic effects of the disease. The condition remains a leading cause of maternal morbidity and mortality in the U.K. [2], but the fetus may also be severely affected, either by growth restriction due to placental insufficiency or by premature delivery [3].

Pre-eclampsia
In pregnancy, inadequate trophoblast invasion results in high-resistance vessels and placental underperfusion, leading to multiple metabolic changes including hypoxia and oxidative stress, and disturbances in the maternal circulation that result in the systemic abnormalities described above [4]. Plasma from women with pre-eclampsia has biological activity that is not present in plasma from women with normal pregnancy [5–7]. A number of factors that may link abnormal placental development to systemic endothelial dysfunction in PET have been proposed [4], but the primary candidates have been linked to the VEGF (vascular endothelial growth factor) family of proteins and their receptors, in particular sVEGFR1 (soluble VEGF receptor 1), PlGF (placental growth factor) and VEGF-A [8–10].

The VEGF family of proteins and receptors in pre-eclampsia
VEGF was first termed vascular permeability factor when it was partially isolated in ascitic fluid in 1983 due its ability to increase vascular permeability [11]. This family now numbers five members in humans: VEGF-A, -B, -C and -D and PlGF. The most widely studied form, VEGF-A (or simply VEGF), is expressed as numerous isoforms caused by alternative exon splicing, resulting in mature proteins varying from 121 to 206 amino acids. VEGF165 is the dominant angiogenic molecule in physiological and pathological angiogenesis [12]. It is produced by a variety of cells and tissues, including the placental syncytiotrophoblasts and placental endothelial cells [9,10], and its production is generally increased by hypoxia [13,14]. Alternative splicing of VEGF-A can also result in an alternative family of anti-angiogenic isoforms, such as VEGF165b [15] (Figure 1). These isoforms act as weak agonists of VEGFR2, preventing VEGF165 from inducing angiogenesis. Although there is little evidence for a role of...
Figure 1 | Alternative splicing of VEGF-A pre-RNA results in multiple isoforms of two families with alternative terminal exon structures resulting in two different families

Boxes are coding sequence. Lines are untranslated regions (UTRs). Functional domains shown on full-length RNA. Light-coloured boxes indicate predicted mRNA species (not yet described).

VEGF-C and -D in pre-eclampsia, PlGF is also integrally linked, as it is predominantly produced by the placenta, is significantly down-regulated in pre-eclampsia [16], and its down-regulation occurs under hypoxia [17] and at or even before the onset of pre-eclamptic symptoms [8], implying that it could be a contributory factor to the symptomology of the disease.

**VEGFRs**

VEGFs bind VEGFR1, VEGFR2 and VEGFR3, tyrosine kinase receptors through which their signal transduction can be initiated. VEGF-A binds VEGFR1 and VEGFR2, but, although it has a higher affinity for VEGFR1, the VEGF165 isoform acts mainly through VEGFR2 to initiate increased permeability, angiogenesis and vasodilatation [18]. In contrast, VEGF165b is a weak agonist for VEGFR2 and prevents VEGF165-mediated signalling that results in angiogenesis [19–21], but use of receptor-specific antagonists has shown that VEGF165b increases hydraulic conductivity ($L_p$, the permeability of vessels to convective water flux) through VEGFR1 [22]. PlGF only binds VEGFR1, but does not increase $L_p$ in the same system [23]. VEGFRs are also produced as alternative splice variants. VEGFR1 [24] and VEGFR2 [25] have secreted splice variants that lack the transmembrane and cytoplasmic domains. These soluble VEGFRs bind to VEGFs and inactivate them [24].

**VEGF and VEGFR expression in PET**

**VEGF**

The properties of VEGF led to its investigation as a potential pathophysiological molecule in PET. However, there is a substantial, critical and serious discrepancy in the literature concerning the level of circulating VEGF in pre-eclamptic plasma (Table 1). Abnormally high levels of VEGF in PET plasma and serum [26,27], amniotic fluid [28], umbilical cord serum [29] and urine [30] have been described using RIA or cEIA (competitive enzyme immunoassay), whereas commercial VEGF ELISAs show a decrease in VEGF levels [31–33]. This discrepancy has been proposed to be due to interference by VEGF-binding molecules in the ELISA [34], but whether this results in reduced biologically active VEGF has only ever been an assumption [35,36], and is contradicted by studies demonstrating biological activity of VEGF in pre-eclamptic plasma [6,7,37]. For example, a polyclonal VEGF RIA showed that total circulating VEGF concentrations in women who develop PET is 34 ng/ml
Table 1 | Measured concentrations of VEGF, sVEGFR1 and PlGF in plasma or serum from women with pre-eclampsia compared with controls

References are up to 2005. No new information since 2005 has described VEGF-A, PlGF or sVEGFR1 levels in any more detail except for [40], which describes the VEGF165b levels. BDL, reported as below detection limit.

<table>
<thead>
<tr>
<th>VEGF-A (ng/ml)</th>
<th>sVEGFR1 (ng/ml)</th>
<th>PlGF (ng/ml)</th>
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<tbody>
<tr>
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<td>Normal</td>
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<tr>
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* Estimated from graph.

cOMPARED WITH normal controls (13 ng/ml) [34,38]. Post-delivery VEGF concentrations fall in both PET and control women, suggesting that the placenta is the main source of VEGF production [38]. VEGF mRNA studies have been inconclusive and contradictory [9,10,39]. We showed recently that circulating VEGF165b in patients with pre-eclampsia was raised during pregnancy, but that it was not significantly higher in PET plasma than in normal pregnancy at term, although there was a lack of up-regulation earlier on in pregnancies that subsequently developed pre-eclampsia [40]. When both VEGF165b and total VEGF were estimated, normal pregnancies had VEGF levels that were calculated (from total VEGF and VEGF165b measurements) at ~50% VEGF165b and 50% VEGF165, whereas in the small number of PET patients in which both could be reliably estimated, this was estimated at 69% VEGF165b and 31% VEGF165 [40]. However, there is still a need to determine the relative levels of the two sets of isoforms, using assays that are not interfered with by circulating macromolecules.

sVEGFR
Both VEGFRs have soluble splice variants. Although soluble VEGFR2 levels do not change in pre-eclamptic plasma (5–6 ng/ml) [41], higher circulating levels of sVEGFR1 occur in PET plasma and serum than in normal pregnancy [8,42,43]. In animal models, adenovirus-mediated overexpression of sVEGFR1 results in pre-eclamptic like symptoms (proteinuria and hypertension) in pregnant animals. This led to the widespread, but untested, concept in the field that sVEGFR1 may be causal for pre-eclampsia in humans. However, there are a number of significant critical and serious inconsistencies in the interpretation and extension to human disease from the experimental design of these animal studies. First, levels of sVEGFR1 (388 ng/ml) [35] in the pregnant animal models were two orders of magnitude greater than that seen in humans in pregnancy (3.1–4.3 ng/ml or 25 pM) [8,44]. Secondly, in humans, the VEGF-A levels are ~800 pM during PET, as measured by RIA [44]. As VEGF–sVEGFR1 binding is equimolar, the sVEGFR1 levels should not be high enough to affect VEGF levels in humans, whereas in the animal model, the sVEGFR1 levels would exceed the VEGF-A levels 3-fold. In fact, in one study where ‘free’ (measured by ELISA) and ‘total’ (measured by cEIA) VEGF levels and sVEGFR1 levels were measured, the molar ratio of total VEGF-A to sVEGFR1 levels was 25-fold (i.e. VEGF-A levels were 547 pM, and the sVEGFR1 levels were 21.9 pM) [42]. Thus with these numbers, it is not possible for sVEGFR1 to bind all of the VEGF. Thus, although there is no doubt that sVEGFR1 is raised in pre-eclampsia, it cannot account for the reduced VEGF levels seen by ELISA, even when combined with sVEGFR2, and the ELISA must be being interfered with by other molecules that bind VEGF, which may not affect VEGF activity on its receptor. In fact, it has been demonstrated clearly that VEGF-A can bind both covalently [45], and, predominantly, non-covalently to α2-macroglobulin, and that this latter interaction does not affect its ability to activate the receptor [46]. As α2-macroglobulin is one of the most common circulating proteins in plasma, and
is found at concentrations far exceeding that of VEGF (2–4 mg/ml, compared with 5–25 ng/ml), it is much more likely that ‘bound’ VEGF is bound, not to sVEGFR1, which might inactivate it, but to α2-macroglobulin, which would not. In contrast, the raised levels of sVEGFR1 could affect the levels of free PlGF.

**PlGF**

PlGF is alternatively spliced to form four mRNA species (PlGF1–PlGF4), of which only PlGF2 has been found in mouse [47–49], an interesting caveat to rodent models of pre-eclampsia. PlGF-2 and PlGF-4 contain an additional 21-amino-acid insert that encodes a heparin-binding domain, resulting in cell association. Circulating PlGF in humans is predominantly PlGF-1 and its levels are tightly linked to human pre-eclampsia in that they have been shown to be significantly reduced [16,40,50–52]. However, the role of PlGF in the pathogenesis of PET is not known partly due to a lack of understanding of its physiological actions in general.

**Biological activity of PET plasma**

It has been postulated for many years that circulating factors altered in pre-eclampsia affect endothelial function [53–55]. Endothelial cells in culture can be stimulated by plasma from women with PET [7,54], and some effects are blocked by VEGF-neutralizing antibodies [7], implicating VEGF as a bioactive molecule in pre-eclampsia. Experiments using myometrial resistance vessels obtained at Caesarean section using wire myography [56] showed that plasma from women with PET, but not normotensive pregnancies reduced endothelium-dependent relaxation. This response did not occur when the plasma was incubated with anti-VEGF antibodies [56]. Many downstream pathways have now been shown to be activated by PET plasma, and many pathways have been proposed to be responsible for the symptoms of pre-eclampsia. These include the production or up-regulation of superoxide [57], MCP1 (monocyte chemoattractant protein 1) and IL (interleukin)-6 [58], IL-8 [59], P- and E-selectin and VCAM (vascular cell adhesion molecule) [60], PGI2 (prostaglandin I2) [61] and cadherin rearrangement [62]. Interestingly, all of these have been shown to be up-regulated or induced by VEGF [63–69].

Thus VEGF may be important in mediating the endothelial response that occurs in PET. In 2004, using an amphibian model to identify permeabilizing agents in human plasma, we confirmed that a high-molecular-mass molecule (>12 kDa) circulating in human plasma from severe pre-eclamptic patients resulted in a transient rapid increase in the L_p of the vessel wall [5] that was qualitatively similar to that seen by VEGF-A in the same system [70]. Although there is no suggestion that the permeability increase that we see in this animal model relates to the symptoms of pre-eclampsia, understanding the mechanisms through which it works may give us a potential mechanism for the endothelial dysfunction induced by pre-eclamptic plasma on human endothelium, and thus a hypothesis to test which may reveal key mechanisms underlying the pathogenesis of pre-eclampsia.

Of interest was a subsequent study that confirmed the biological activity of VEGF in pre-eclamptic plasma. The transient increase in permeability was blocked by VEGF-neutralizing antibodies, and was inhibited by a concentration of a VEGFR TKI (tyrosine kinase inhibitor) (SU5416) shown previously to block the VEGF165_b effect, but not the effect of VEGF165, on these vessels [22]. Moreover, a concentration of an inhibitor shown previously to block VEGF165-mediated permeability through inhibiting VEGFR2 phosphorylation (ZM323881) in this system [71] did not block the pre-eclamptic plasma-mediated permeability response. Of particular interest was the effect of a specific antibody against VEGF165_b [19], which blocks VEGF165_b-mediated inhibition of VEGF165-induced migration of endothelial cells and VEGF165_b-induced cytoprotection of endothelial and epithelial cells [72]. The permeability increase was blocked by this VEGF165_b-neutralizing antibody. This was an extremely surprising finding and difficult to reconcile with the lack of any increase in VEGF165_b in PET plasma compared with normotensive plasma at term when these samples were taken.

The increase in permeability in this model induced by term pre-eclamptic plasma is clearly an effect of VEGF, but not simply due to excess VEGF165_b. There are a number of possible mechanisms, but to outline these, it is necessary to examine the mechanisms of action of the three major contributors involved: VEGF, sVEGFR1 and PlGF.

**Mechanisms of actions of VEGF, PlGF and sVEGFR1**

**VEGF-A**

The mechanisms of action of the pro-angiogenic isoforms of VEGF have been widely studied. VEGF165 acts through VEGFR2, resulting in a transient calcium influx, and a rapid transient increase in permeability followed by a sustained increase due to one or more of a combination of fenestrations, vesiculovacuolar organelles, endothelial gaps, tight junction and adherent junction disassembly [73]. The mechanism of the action of VEGF165_b is less well described, but VEGF165_b results in a rapid transient increase in L_p that is smaller in magnitude but greater in potency than VEGF165, probably acting through VEGFR1 not VEGFR2 [22].

**PlGF**

PlGF acts through VEGFR1, but its downstream signalling is still not well understood and there are conflicting reports of its biological activity. PlGF-1 does not cause a transient increase in L_p, in the same model used to investigate VEGF165_b, VEGF165 and VEGF-C signalling [23], although studies using PlGF2-knockout mice point to a rather more complex role, as these mice have reduced ‘leak’ in response to VEGF-A [74]. However, PlGF-1 has been shown to be a potent vasodilator [75], particularly in uterine arteries. Blockade of PlGF-1 in pregnancy would therefore result in...
increased vascular tone, and hence hypertension as seen in pre-eclampsia.

sVEGFR1
The role of VEGFR1 has generally been characterized as a decoy receptor. It has been hypothesized that high levels of sVEGFR1 antagonize the effects of VEGF and PlGF on placental development, vascularization and maternal endothelial cell function [42], and thus the increase in sVEGFR1 in maternal plasma has been postulated to inhibit VEGF-A. However, VEGF-A induces increased vascular permeability (and hence oedema), vasodilatation and angiogenesis [70,76]. Increased sVEGFR1 should therefore prevent the permeability responses of pre-eclamptic plasma, not induce them. Thus increased sVEGFR1 acting on VEGF-A by itself does not explain the symptoms of pre-eclampsia or experimental findings of the effect of PET plasma, but sVEGFR1 acting on PlGF, and removing the inhibition of VEGF165b, would explain the symptoms.

However, this scenario is not only unproven, but theoretically difficult to reconcile with measurements made. PlGF binds to sVEGFR1 with the same affinity as VEGF165 (PlGF competes with binding of 10 ng/ml radiolabelled VEGF165 with an IC50 of ~10 ng/ml) [77]. However, circulating levels of PlGF are an order of magnitude lower than VEGF, and therefore most of the sVEGFR1 should be bound to VEGF-A not PlGF. This discrepancy has yet to be resolved, but it is possible that circulating levels of PlGF are lower in pre-eclamptic plasma because PlGF may be secreted at a lower level in pre-eclamptic pregnancies, and sVEGFR1 binding is irrelevant.

Thus plasma from normal pregnancy shows no biological activity on endothelium of intact vessels. In contrast, PET plasma increases permeability and blocks vasodilatation in the same models, and, in the permeability model, this is blocked by a VEGF165b-neutralizing antibody and by VEGFR1 kinase inhibitors. There is no increased VEGF165b, but reduced PlGF and increased sVEGFR1 and VEGF165b/VEGF165 Ratio, so an interplay between PlGF, VEGF165, VEGF165b and sVEGFR1 is hypothesized. This was tested by incubating pre-eclamptic plasma with PlGF, which blocked the biological response.

In summary, the current models of pre-eclampsia based on the role of sVEGFR1 affecting vascular permeability, hypertension and proteinuria do not appear to take into account the findings in the literature of the biologically active VEGF levels in women with pre-eclampsia. Alternative models involving sVEGFR1 competition of PI GF-mediated repression of VEGF activity, or sVEGFR1-independent mechanisms need to be tested, particularly as it is the low availability of PlGF that may be the key pathological and treatable disorder of pre-eclampsia. Understanding this interplay between PlGF, sVEGFR1 and VEGF may therefore reveal mechanisms through which PET pathophysiology occurs in humans.

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References

19 Woolard, J., Wang, W.Y., Bevan, H.S., Qiu, Y., Morbidelli, L.,

21 Cebe Suarez, S., Pieren, M., Cariolato, L., Arn, S., Hoffmann, U., Bogucki,

24 Kendall, R.L. and Thomas, K.A. (1993) Inhibition of vascular endothelial


25 Albuquerque, R.J., Hayashi, T., Cho, W.G., Kleinman, M.E., Dridi, S.,

29 Galazios, G., Papazoglou, D., Giagloglou, K., Vassaras, G., Koutlaki, N. and


37 Bills, V.L., Salmon, A.H., Harper, S.J., Overton, T.G., Neal, C.R., Jeffery, B.,


regulation due to the VEGF165b splice variant in pre-eclampsia. BJOG

38 Hunter, A., Aitkenhead, M., Caldwell, C., McCracken, G., Wilson, D. and

McClure, N. (2000) Serum levels of vascular endothelial growth factor

in preeclampsia and non-pregnant women. Hypertension 36, 965-969


41 Manuyama, H., Suzuki, N., Nakahaski, H., Masumoto, A., Takeshi, Y. and


42 Tsatsaris, V., Goffen, F., Munaut, C., Brichant, J.F., Pignon, M.R., Noel, A.,


Overexpression of the soluble vascular endothelial growth factor


43 Koga, K., Osuga, Y., Yoshino, O., Hirata, Y., Ruimeng, X., Hirata, T.,


44 McKeeman, G.C., Ardill, J.E., Caldwell, C.M., Hunter, A.J. and McClure, N.

(2004) Soluble vascular endothelial growth factor receptor-1 (sFlt-1) is increased throughout gestation in patients who have preeclampsia develop. Am. J. Obstet. Gynecol. 191, 1240-1246

growth factor is inactivated by binding to α2-macroglobulin and the

binding is inhibited by heparin. J. Biol. Chem. 268, 7685-7691

The conformation-dependent interaction of α2-macroglobulin with

vascular endothelial growth factor: a novel mechanism of

α2-macroglobulin/growth factor binding. J. Biol. Chem. 275,

26806-26811

47 Maglione, D., Guerrieri, V., Vigletto, G., Ferraro, M.G., Apeilikova, O.,

Altal, K., Del Vecchio, S., Lei, K.J., Chou, J.Y. and Persico, M.G. (1993)

Two alternative mRNAs coding for the angiogenic factor, placenta
growth factor (PIGF), are transcribed from a single gene of chromosome

14. Oncogene 8, 925-931


identification and characterization of a novel isoform generated by RNA


human trophoblast and endothelial cells. J. Reprod. Immunol. 60, 53-60


Maternal plasma levels of cytokines in normal and preeclampsia

pregnancies and their relationship with diastolic blood pressure and

uroconectin levels. Acta Obstet. Gynecol. Scand. 82, 797-802

51 Bersinger, N.A. and Odegard, R.A. (2005) Serum levels of macrophage

colony stimulating, vascular endothelial, and placenta growth factor

in relation to later clinical onset of pre-eclampsia and a small-for-gestational age birth. Am. J. Reprod. Immunol. 54, 77-83

52 Thadhani, R., Mutter, W.P., Wolf, M., Levine, R.J., Taylor, R.N., Sukhatme,


regulation due to the VEGF165 b splice variant in pre-eclampsia. BJOG

111, 269-276


55 Taylor, R.N., Casal, D.C., Jones, L.A., Varma, M., Martin, J.N. and


Preeclamptic sera stimulate increased platelet-derived growth factor


