



Soluble FLT1 sensitizes endothelial cells to inflammatory cytokines by antagonizing VEGF receptor-mediated signalling

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Aims

Pre-eclampsia affects 5–7% of pregnancies, and is a major cause of maternal and foetal death. Elevated serum levels of placentally derived splice variants of the vascular endothelial growth factor (VEGF) receptor, soluble fms-like tyrosine kinase-1 (sFLT1), are strongly implicated in the pathogenesis but, as yet, no underlying mechanism has been described. An excessive inflammatory-like response is thought to contribute to the maternal endothelial cell dysfunction that characterizes pre-eclampsia. We hypothesized that sFLT1 antagonizes autocrine VEGF-A signalling, rendering endothelial cells more sensitive to pro-inflammatory factors also released by the placenta. We tested this by manipulating VEGF receptor signalling and treating endothelial cells with low doses of tumour necrosis factor- α (TNF- α).

Methods and results

Application of recombinant sFLT1 alone did not activate human umbilical vein endothelial cells (HUVECs). However, antagonizing the autocrine actions of endothelial VEGF-A and/or placenta growth factor (PlGF) by pre-incubation with recombinant sFLT1, anti-FLT1, anti-VEGF receptor 2 (KDR), anti-VEGF-A, VEGF receptor tyrosine kinase inhibitor SU5614, or knocking-down *FLT1* or *KDR* transcripts rendered cells more sensitive to low doses of TNF- α . Each treatment increased activation, as measured by increases in endothelial intercellular adhesion molecule 1 (ICAM1), vascular cell adhesion molecule 1 (VCAM1), endothelin 1 (ET-1), von Willebrand factor (vWF), and leucocyte adhesion, and led to reduction in AKT Ser⁴⁷³ and endothelial nitric oxide synthase (eNOS) Ser¹¹⁷⁷ phosphorylation.

Conclusions

Our data describe a mechanism by which sFLT1 sensitizes endothelial cells to pro-inflammatory factors, providing an explanation for how placental stress may precipitate the pre-eclamptic syndrome.

Keywords

Pre-eclampsia • sFLT1 • Endothelial dysfunction

1. Introduction

Pre-eclampsia is a pregnancy-specific disorder characterized by *de novo* hypertension and proteinuria during the third trimester. The condition affects 5–7% of pregnancies worldwide and is a major cause of maternal death, estimated to be in excess of 63 000 a year.¹ Clinical presentation is heterogeneous, but two forms of the syndrome are now recognized; placental pre-eclampsia

which occurs secondary to defective placentation, and maternal pre-eclampsia which arises due to a maternal susceptibility to factors emanating from a normal placenta.² The pathophysiology of placental pre-eclampsia is generally attributed to abnormal conversion of the maternal spiral arteries supplying the placenta, and subsequent placental malperfusion. The malperfusion is thought to result in placental oxidative stress and release of a complex mix of factors, including pro-inflammatory cytokines, apoptotic

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debris, and angiogenic regulators, into the maternal circulation. This release is believed to culminate in an enhanced maternal inflammatory response and systemic endothelial dysfunction leading to the maternal syndrome.^{2–4} To date, however, no single factor has been identified that can be considered causal of the syndrome.²

A significant step in unravelling this complexity was made when Maynard et al.⁵ showed that inhibition of vascular endothelial growth factor (VEGF-A) and placenta growth factor (PlGF) action through over-expression of soluble fms-like tyrosine kinase-1 (sFLT1) causes a pre-eclampsia-like syndrome in pregnant rats. Soluble fms-like tyrosine kinase-1 (sFLT1) is formed by alternative splicing of the pre-mRNA encoding the full-length-signalling VEGF-R1 (FLT1) receptor, and lacks the cytoplasmic and transmembrane domains. sFLT1 antagonizes the pro-angiogenic molecules VEGF-A and PlGF.⁶ In the last 2 months of all pregnancies, maternal circulating levels of sFLT1 increase, and those of free PlGF decrease. These changes are more pronounced in women who develop pre-eclampsia, and occur up to 5 weeks before the onset of clinical symptoms.^{5,7} Levels of sFLT1 are also raised in pre-eclamptic placentas at delivery.^{5,8} Animal studies provide strong evidence linking sFLT1 to the pathogenesis of pre-eclampsia. Treatment of pregnant rats with an adenovirus encoding sFLT1 induces hypertension, proteinuria, and glomerular endotheliosis, the classical lesion of pre-eclampsia.⁵ Additionally, loss of a single VEGF allele in murine glomerular podocytes results in proteinuria and glomerular endotheliosis.⁹ Similarly, administration of a therapeutic VEGF-A neutralizing antibody (Bevacizumab, Avastin Genentech) leads to renal pathology which can be replicated in adult animals by conditional gene targeting.¹⁰ These studies all demonstrate that abnormal inhibition of endogenous VEGF-A activity can induce maternal pre-eclampsia-like symptoms.

Equally, placental malperfusion appears an adequate stimulus for the release of sFLT1. Uteroplacental ischaemia leads to the elevation of placental and circulating levels and proteinuria,¹¹ while the repetitive restriction in blood flow during labour is associated with increased placental sFLT1 mRNA and protein.¹² sFLT1 secretion is also induced by hypoxia in primary cytotrophoblast cells,¹³ and by hypoxia,¹⁴ hypoxia-reoxygenation,¹⁵ and tumour necrosis factor- α (TNF- α)¹⁴ in placental explants.

Despite this strong evidence, the mechanism by which excessive sFLT1 causes maternal endothelium activation is not known. Numerous papers simply state that sFLT1 is elevated, leading to 'endothelial dysfunction'.^{7,16,17} There is no doubt that endothelial dysfunction does occur in pre-eclamptic patients,¹⁸ as evidenced by increased markers of activation, including intercellular adhesion molecule 1 (ICAM1),¹⁹ vascular cell adhesion molecule 1 (VCAM1),²⁰ endothelin 1 (ET-1),²¹ von Willebrand factor (vWF),²² and thrombomodulin.²³ Whether this is due to reduced bioavailability of circulating VEGF-A in the maternal blood due to binding with sFLT1, or to competitive inhibition of full-length VEGF receptor signalling on the endothelial cell surface is not known. The finding that sFLT1 acts synergistically with the soluble TGF- β co-receptor, endoglin (sENG),¹⁶ to cause endothelial cell activation suggests the latter may be the case. Additionally, sFLT1 might play a role in reducing the bioavailability of nitric oxide (NO) by antagonizing the effects of VEGF-A, which up-regulates endothelial nitric oxide synthase (eNOS) mRNA and protein,²⁴ and sFLT1 might also reduce VEGF-induced c¹¹⁷⁹ phosphorylation.²⁵

A robust inflammatory response is a feature of all pregnancies, and is further enhanced in pre-eclamptic pregnancies.²⁶ An excessive inflammatory response has been proposed to be the driving force

that contributes to endothelial cell dysfunction and the clinical syndrome of pre-eclampsia.²⁷ We therefore hypothesized that sFLT1 antagonizes autocrine VEGF-A signalling at the endothelial receptor level, so rendering endothelial cells more sensitive to pro-inflammatory factors released by the stressed placenta. We tested this hypothesis by manipulating VEGF receptor signalling by a wide range of techniques and treating the endothelial cells with low doses of TNF- α .

2. Methods

2.1 Culture and treatment of HUVECs

Umbilical cords were collected from uncomplicated pregnancies with the informed written consent of the patients and permission of the Local Research Ethics Committee (ethical number 03/360). The investigation also conforms with the principles outlined in the Declaration of Helsinki. Pools of human umbilical vein endothelial cells (HUVECs) were seeded into appropriate culture wells and pre-treated the next day for 24 h with sFLT1 (50–250 ng/mL) and/or neutralizing antibodies, i.e. anti-FLT1 (5 μ g/mL), anti-KDR (1 μ g/mL), and anti-VEGF-A (5 μ g/mL). On Day 3, sFLT1 was again added to the medium and, where applicable, cells were pre-incubated with SU5614 (5 μ M), LY294002 (0.1–10 μ M), or PF573228 (0.1–10 μ M) for 30 min. A low dose of TNF- α (0.5 ng/mL) was added and cells were incubated for a further 6 h.

2.2 Cell adhesion assay

Pooled HUVECs were seeded into 96-well plates and treated with recombinant sFLT1 and TNF- α in quadruplicate, as described above. In the meantime, differentiated HL60 cells (ECACC European Collection of Cell Cultures) were spun down and resuspended at 1×10^6 /mL in phosphate buffered saline (PBS). Cells were labelled by incubation at 37°C for 20 min in cell tracker green CMFDA (5-chloromethylfluorescein diacetate) dye (2.5 μ M, Invitrogen, Molecular Probes, Paisley, UK). Labelled cells and an aliquot of retained unlabelled HL60 cells were washed and resuspended at 5×10^5 /mL in Dulbecco's modified Eagle medium. In the last hour of the HUVEC TNF- α incubation, 100 μ L of HL60 cell suspension was added to each well. The 96-well plate was incubated for the remaining hour at 37°C, followed by aspiration of medium and three washes with 200 μ L PBS. Fluorescence was measured using FLUOstar Optima Fluorimeter (BMG Labtech, Aylesbury, UK).

2.3 Transfection with siRNAs

Pooled HUVECs were seeded into T25 flasks at 5.0×10^5 cells per flask and incubated overnight. The T25 flasks were incubated with a mixture of siRNA (100 nM final concentration), medium and siFECTamine buffer (ICVEC, London, UK) for 3 h at 37°C, washed and incubated for further 3 h at 37°C. Cells were trypsinized, counted, and seeded into 96-wells or 12-wells at 1×10^4 and 5×10^4 , respectively, and cultured for 24–48 h. Transfected HUVECs were treated with sFLT1 and the HL60 adhesion assay and western blots (see Supplementary material online, *Methods*) performed. siRNA-mediated knock-down efficiency was checked by quantitative RT-PCR as previously described.¹²

2.4 Immunofluorescent cytochemistry and colorimetric immunohistochemistry

For immunofluorescence, cells were seeded onto gelatin-coated 13-mm glass cover slips. At the end of the experiment, cells were fixed in 2% paraformaldehyde at 4°C for 30 min. Cover slips were washed in PBS and, if not stained immediately, stored in PBS at 4°C until required. Cells were immunostained according to the protocol described previously,¹² which involved the use of Alexa 488 or Alexa 568 secondary fluorescent antibodies (Invitrogen) and mounting in Vectashield mounting medium

containing DNA stain DAPI (4',6-diamidino-2-phenylindole; Vector, UK). Images were captured and quantified using a Leica confocal microscope (LeicaTCS-NT, Leica Instruments GmbH, Germany). All comparable images, i.e. controls and treated cells, were captured at the same laser and pinhole setting. At least six fields per treatment were captured and quantified using the Leica software. Values for each treatment were expressed as cell fluorescence per micrometre. All experiments were repeated at least three times. We validated the immunofluorescent quantification using FACS analysis (see Supplementary material online, *Methods and Figure S1*).

A previously published protocol was followed for immunostaining of paraffin-embedded placental sections,¹² which involved the use of Vectastain Elite ABC kits (Vector Laboratories) and SigmaFast DAB (diaminobenzidine; Sigma, Poole, UK).

2.5 RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was isolated from HUVECs using Trizol reagent. The RNA was quantified by spectrophotometry (Nanodrop Technologies, Rockland, DE, USA). In brief, 20 μ g of total RNA from each sample was reverse transcribed using a master mix containing SuperScript II Reverse Transcriptase (Invitrogen). The ABI PRISM 7700 Sequence Detection System (TaqMan) was used to perform real-time PCR according to the manufacturer's protocols. 'Ct' values for each transcript were compared with those for 18S rRNA. All primers and probes were obtained from Applied Biosystems (ABI, Warrington, UK) 'Assays-on-Demand' and used a 5' FAM reporter and 3' non-fluorescent minor groove binder.

2.6 Statistical analysis

All data are presented as means \pm SEM. Statistical analysis was performed using Statview (SAS Institute, Inc., Cary, NC, USA). Measurements were analysed using repeated measures ANOVA. Differences between two groups were evaluated using Fisher's Protected Least Significant Difference (PLSD) analysis. In all cases results were considered significant at $P < 0.05$.

3. Results

3.1 sFLT1 increases activation of HUVECs in the presence of low doses of TNF- α

Activation of HUVECs was assessed by several means, including adhesion of fluorescently-labelled HL60 cells, expression of ICAM1, VCAM1, vWF, and ET-1. sFLT1 treatment alone did not alter HL60 adhesion nor expression of any of the above markers (*Figure 1*, Supplementary materials online, *Figure S2*). However, pre-treatment with increasing doses of sFLT1 followed by stimulation with a low dose of TNF- α (0.5 ng/mL) led to a significant increase in HL60 adhesion (*Figure 1A*), and an increase in ICAM1, VCAM1, vWF, and ET-1 (*Figure 1B–E*, Supplementary material online, *Figure S2*), compared with TNF- α treatment alone. On the basis of the dose response, we chose the concentration of 100 ng/mL of recombinant sFLT1 in subsequent experiments.

3.2 The effect of sFLT1 can be mimicked at the RNA and protein level

We speculated that the effects of sFLT1 could be attributed to reduced availability of autocrine VEGF-A and/or PIGF, or interference with FLT1/KDR signalling. VEGF acts through two high-affinity receptor tyrosine kinases, FLT1 and KDR, both of which are present in normal endothelial cells and are up-regulated during angiogenesis.

We thus attempted to mimic the effects of sFLT1 by disrupting FLT1/KDR signalling at the RNA and protein levels. siRNA was used to knock down *FLT1* and *KDR* transcripts, while anti-KDR, FLT1, and VEGF-A neutralizing antibodies and the SU5614 VEGF receptor tyrosine kinase inhibitor were used to block receptor binding and activity, respectively. TNF- α treatment of HUVECs transfected with siFLT1 or siKDR (pools of four oligonucleotides) led to a significant enhancement of cell adhesion, compared with cells transfected with a control siRNA and treated with TNF- α . In addition, treatment of the transfected cells with recombinant sFLT1 did not further increase the TNF- α -induced adhesive capacity of these cells (*Figure 2A*). Transfection of HUVECs with siTNF-RSF1A served as a control for the cell adhesion assay, and, as expected, siTNF-RSF1A-transfected cells showed very little increase in HL60 adhesion upon TNF- α stimulation. The adhesion of HL60 leucocytes was ICAM1-dependent. ICAM1 is an endothelial adhesion molecule for neutrophils. siRNA-mediated ICAM1 knock-down greatly reduced TNF- α -stimulated adhesion, similar to the response of siTNFR-SF1A-transfected cells (*Figure 2A*). Additionally, we tested the specificity of the siRNA pools using individual duplexes for siKDR and siFLT1. Transfection with individual duplexes increased the sensitivity of HUVECs to the TNF- α treatment and the results were comparable with those obtained with the siRNA pool (see Supplementary material online, *Figure S3*). The efficacy of the knockdown was demonstrated by quantitative RT-PCR and was $>50\%$ (see Supplementary material online, *Figure S4*). Simultaneous siRNA-mediated knock-down of both VEGF receptors resulted in similar enhancement of HL60 adhesion to that observed when knocking down the individual receptors (see Supplementary material online, *Figure S5*).

Similar increases in cell adhesion upon treatment with TNF- α were observed when HUVECs were pre-treated with neutralizing antibodies to KDR, FLT1, and VEGF-A, or with the VEGF receptor kinase inhibitor SU5614 (*Figure 2B*). Addition of recombinant sFLT1 in conjunction with TNF- α did not further increase HL60 adhesion following any of these pre-treatments. The increase in adhesion of HL60 cells to HUVECs concomitantly treated with TNF- α and neutralizing antibodies or SU5614 was associated with a significant increase in ICAM1, VCAM1, vWF, and ET-1 (*Figures 2C, 3A–D*). Based on these results, which indicate that inhibition/neutralization of either KDR or FLT1 leads to similar effects as those induced by sFLT1 alone, we inferred that cross-talk was occurring between these two receptors. Immunoprecipitation of control or sFLT1-treated cell lysates with anti-KDR and subsequent detection with anti-FLT1 confirmed this. A band corresponding to FLT1 was detected in both cell samples precipitated with anti-KDR and an additional band corresponding to sFLT1 was present in the sFLT1-treated cell sample (see Supplementary material online, *Figure S6*). Thus the two receptors are able to form a heterodimer.

3.3 Inhibition of the AKT pathway at protein or RNA level makes HUVECs more sensitive to TNF- α treatment

We decided to examine the PI3K/AKT pathway as KDR activates the PI3K/AKT pathway, and VEGF-induced AKT activation stimulates eNOS Ser¹¹⁷⁹ phosphorylation, leading to Ca²⁺-independent NO generation.²⁵ In addition, VEGF-A up-regulates eNOS mRNA and protein, thus prolonging the production of the short-lived NO.²⁴

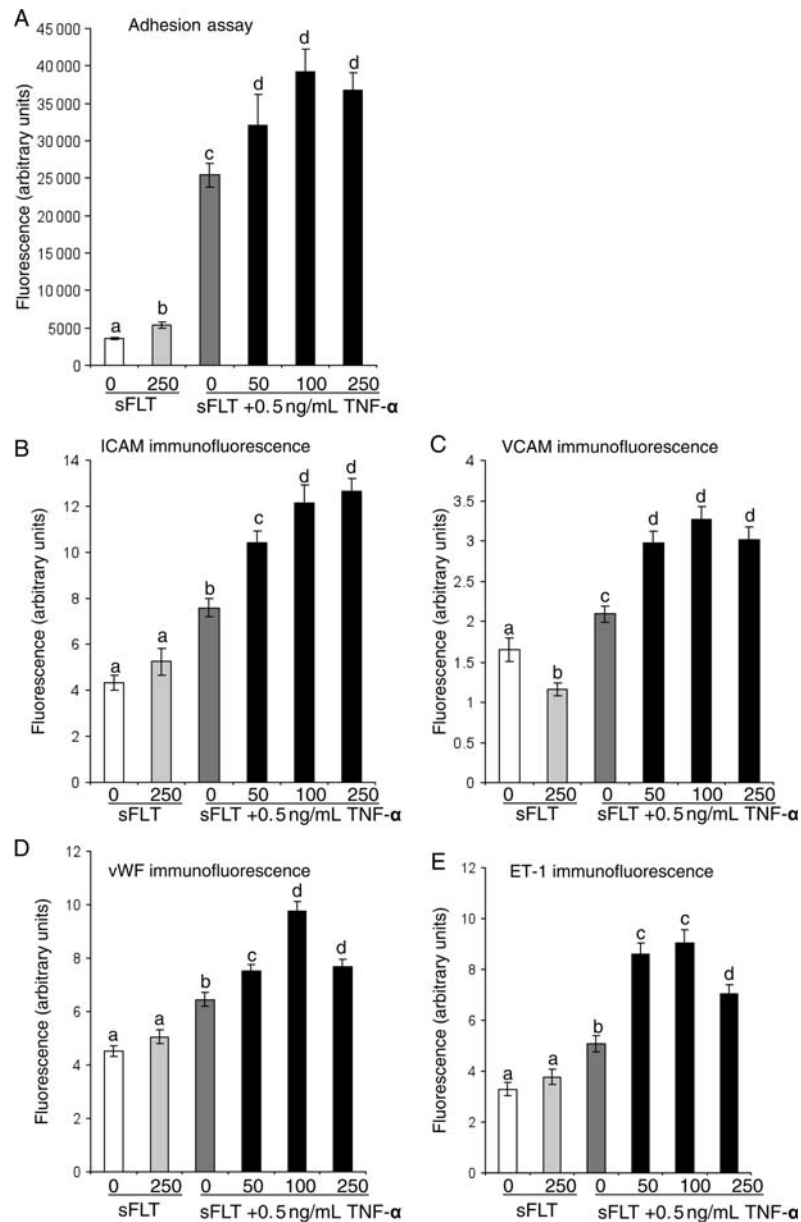


Figure 1 Pre-treatment of HUVECs with sFLT1 significantly increases HL60 leucocyte adhesion, and expression of ICAM1, VCAM1, vWF, and ET-1 upon TNF- α treatment. HUVECs were pre-treated with/without sFLT1 (50–250 ng/mL) for 24 h and subsequently treated with TNF- α for 6 h. Fluorescently labelled HL60 leucocytes were added in the final hour of TNF- α incubation and fluorescence was detected (A). Fixed HUVECs were immunostained against ICAM1 (B), VCAM1 (C), vWF (D), and ET-1 (E), and fluorescence was quantified. Experiments were repeated at least three times. Letters indicate groups significantly different using the PLSD test with $P < 0.05$.

Consistent with the above findings, sFLT1 treatment caused a concentration-dependent decrease in AKT^{Ser473} and eNOS^{Ser1177} phosphorylation. Phosphorylation of eNOS at Ser¹¹⁷⁷ by AKT leads to increased NO production.^{25,28} Thus the concentration-dependent decrease in eNOS phosphorylation seen in our study would be expected to lead to reduced NO production. Total levels of AKT and eNOS remained unchanged (Figure 4A). This decrease was not associated with cell death even at high concentrations (data not shown). Additionally, pre-treatment of HUVECs with recombinant sFLT1, or anti-FLT1 and anti-KDR antibodies resulted in decreased phosphorylation of AKT at Ser⁴⁷³ and decreased phosphorylation of

GSK3 at Ser^{21/9} (data not shown). We thus hypothesized that the increased sensitivity to TNF- α of HUVECs treated with recombinant sFLT1, neutralizing antibodies or transfected with siKDR or siFLT1 could be due to interference with the AKT-signalling pathway. To test this, we transfected HUVECs with siAKT1 or siAKT3 (Figure 4B and C) or pre-treated HUVECs with a PI3-kinase inhibitor, LY294002 (Figure 5), and subsequently tested their sensitivity to TNF- α . We chose siAKT1 and siAKT3 based on the placental expression of these proteins. We found AKT1 in trophoblast and endothelial cells, whereas AKT3 was only detectable in foetal endothelial cells. In contrast, there was no endothelial AKT2, which was only observed in

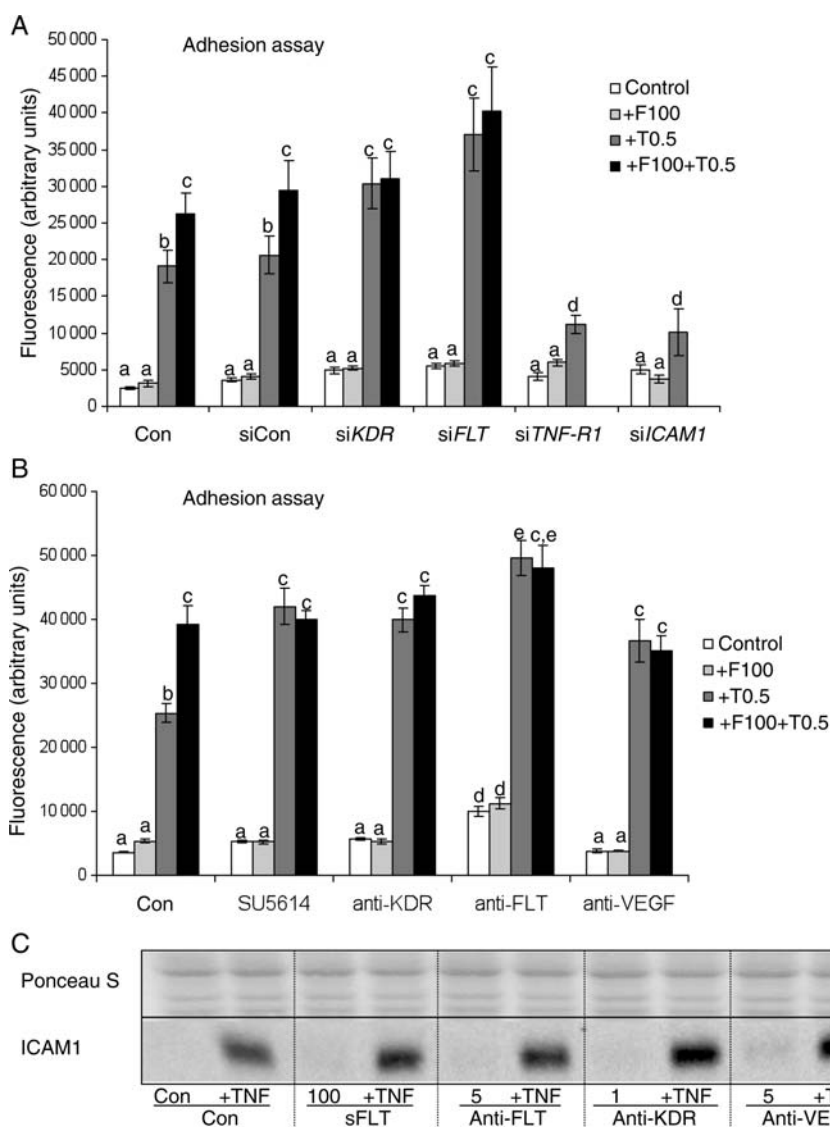


Figure 2 HL60 leucocyte adhesion upon TNF- α treatment is significantly increased in HUVECs transfected with siKDR or siFLT1 or treated with neutralizing antibodies against KDR, FLT1, or VEGF-A or with the VEGF receptor kinase inhibitor SU5614. HUVECs were transfected with siKDR, siFLT1, siTNFR-SF1A, or siICAM1 for 24 h (A), pre-treated with/without sFLT and/or anti-KDR, anti-FLT1 or anti-VEGF-A (B) for 24 h and then treated with TNF- α for 6 h. Fluorescently labelled HL60 leucocytes were added in the final hour of TNF- α incubation and fluorescence was detected. Western blots were run to detect ICAM1 expression (C). These graphs represent one experimental treatment. Experiments were repeated at least three times. Letters indicate groups significantly different using the PLSD test with $P < 0.05$. F100—sFLT1 100 ng/mL; T0.5—TNF- α 0.5 ng/mL.

trophoblast cells (Figure 4D). siAKT1- and siAKT3-mediated knockdown rendered cells more sensitive to TNF- α , as demonstrated by increased adhesion of HL60 leucocytes and increased expression of ICAM1 (Figure 4B and C). Knock-down efficacy was determined by western blotting using anti-AKT1 and AKT3 specific antibodies (Figure 4B). Similar effects could be seen in HUVECs pre-treated with different concentrations of the PI-3 kinase inhibitor, LY294002, which blocks AKT phosphorylation (Figure 5). The increase in HL60 adhesion in the LY294002-treated cells was dose-dependant (Figure 5B).

4. Discussion

We have shown that sFLT1 alone does not induce activation of endothelial cells, but that it acts in synergy with TNF- α to enhance the

latter's pro-inflammatory effects. We hypothesized that the synergistic effect is due to antagonism of autocrine VEGF-A signalling. To confirm this, we mimicked sFLT1 action using neutralizing antibodies to VEGF-A and its two receptors, a receptor tyrosine kinase inhibitor, or small inhibitory RNA sequences directed against FLT1 or KDR. All treatments resulted in greater sensitivity to TNF- α , supporting this hypothesis.

Autocrine VEGF-A signalling is required for endothelial cell survival *in vivo* under non-pathological conditions in a cell-autonomous manner. Endothelial specific ablation of VEGF-A results in progressive endothelial degeneration and sudden death of mutant animals.²⁹ Gerber *et al.*³⁰ showed that autocrine VEGF-A is necessary for the survival of haematopoietic stem cells. In both these cases, paracrine actions of VEGF-A are not sufficient to maintain the target cells. Thus, in pre-eclampsia, the

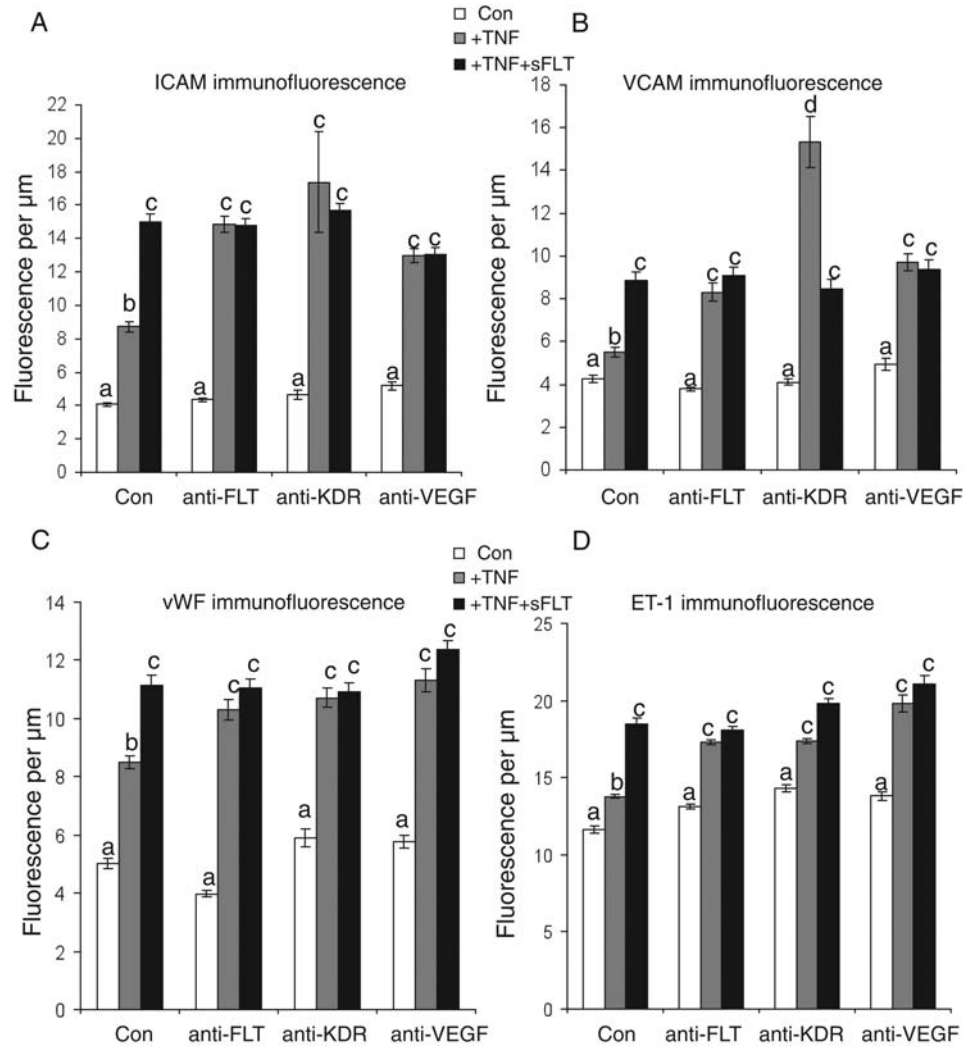


Figure 3 Treatment of HUVECs with neutralizing antibodies against KDR, FLT1, or VEGF-A or with the VEGF receptor kinase inhibitor SU5614 increases the expression of ICAM1, VCAM1, vWF, and ET-1 upon TNF- α treatment. HUVECs were pre-treated with/without sFLT for 24 h, followed by a 30-min pre-treatment with/without anti-KDR, anti-FLT1, or anti-VEGF-A and then treated with TNF- α for 6 h. Fixed HUVECs were immunostained against ICAM1 (A), VCAM1 (B), vWF (C), or ET-1 (D) and immunofluorescence was quantified. Each graph represents one experimental treatment. Experiments were repeated at least three times. Letters indicate groups significantly different using the PLSD test with $P < 0.05$. F100—sFLT1 100 ng/mL; T0.5—TNF- α 0.5 ng/mL.

reduction in such signals caused by sFLT1 binding to most, or even all, of the circulating VEGF-A and/or PlGF would not be expected to alter the essential maintenance signals derived from autocrine (endothelial) VEGF-A. However, if the elevated circulating sFLT1 is able to act in a dominant-negative fashion at the endothelial cell surface then these signals would be blocked. Here, we provide several lines of evidence that support this hypothesis.

FLT1 is known to heterodimerize with KDR³¹ and we have shown that both FLT1 and sFLT1 can be immunoprecipitated with anti-KDR antibodies. Treatment of endothelial cells with agents that block VEGF receptor signal generation (neutralizing antibodies), reduce receptor level (siRNA), or inhibit receptor kinase activity (SU5614) all result in enhanced TNF- α sensitivity, as determined using multiple functional endpoints.

Furthermore, treatment with sFLT1 or FLT1, KDR or VEGF-A neutralizing antibodies led to reduced phosphorylation of AKT and

reduced phosphorylation of eNOS at Ser¹¹⁷⁷. KDR activates the PI3K/AKT pathway, and VEGF-induced AKT activation mediates eNOS Ser¹¹⁷⁹ phosphorylation, leading to Ca²⁺-independent NO generation.²⁵ In addition, VEGF-A up-regulates eNOS mRNA and protein, thus prolonging the production of the short-lived NO.²⁴ NO and PGI₂ (also stimulated by VEGF-A) are mediators of vasodilatation, anti-platelet, and anti-thrombotic effects and inhibitors of leucocyte adhesion.³² Deficient NO production has therefore been proposed as a mechanism linking the elevation of sFLT1 in pre-eclampsia. This is further supported by the negative correlation between plasma or whole blood nitrite concentrations and sFLT1 levels in pre-eclamptic patients, suggesting an inhibitory effect of anti-angiogenic factors on NO formation.³³ VEGF-receptor activity is an important regulator of endothelial NO synthesis.³⁴ Our data suggest for the first time that impaired endothelial function results from sFLT1 blockade of autocrine VEGF-A action and the subsequent

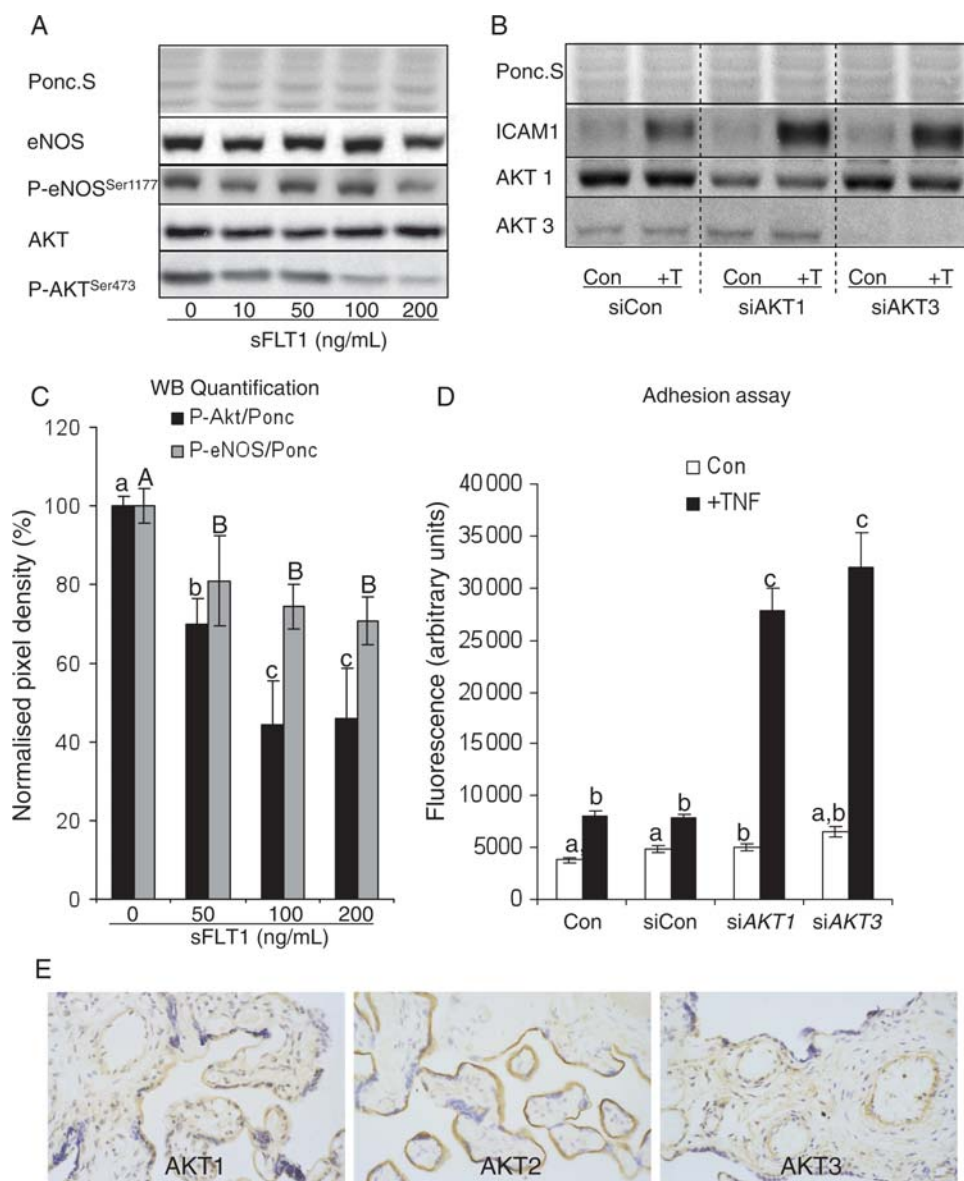
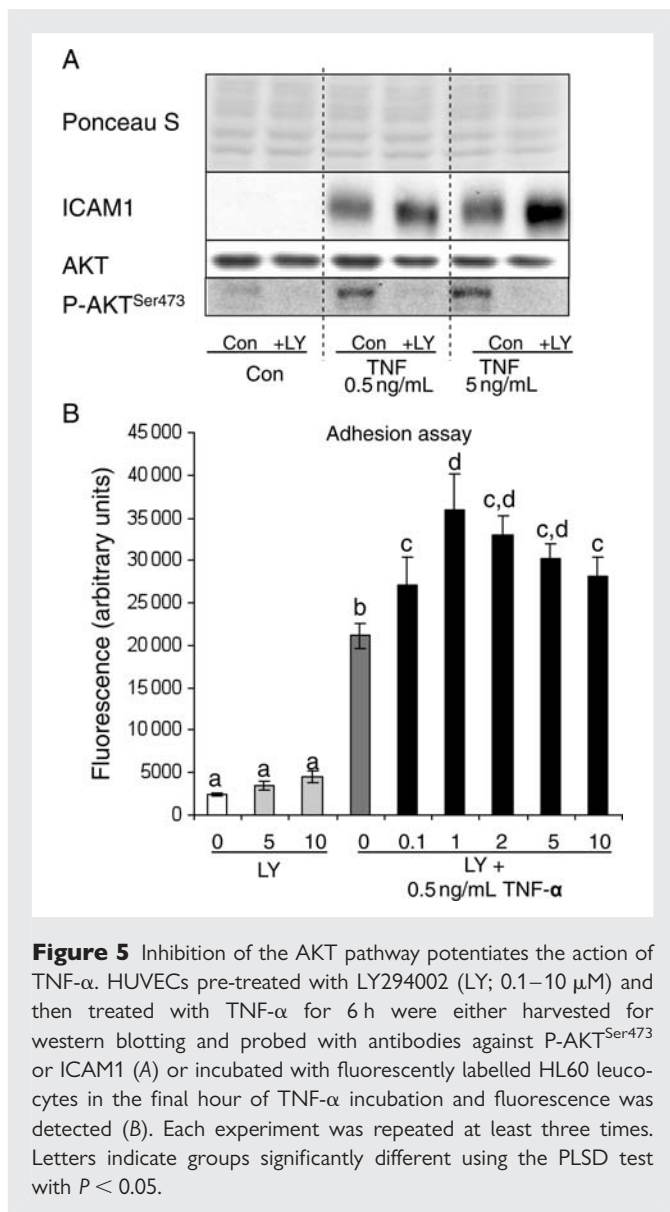


Figure 4 The role of the AKT pathway in sFLT1 signalling. Lysates from HUVECs pre-treated with/without sFLT1 (10–200 ng/mL) for 24 h and subsequently treated with TNF- α for 6 h were immunoblotted with antibodies against P-AKT^{Ser473}, AKT, P-eNOS^{Ser1177}, and eNOS (A) and the signal from three experiments was quantified (C). HUVECs transfected with siAKT1 or siAKT3 for 24 h and then treated with TNF- α for 6 h were either immunoblotted with antibodies against AKT1, AKT3, and ICAM1 (B) or incubated with fluorescently labelled HL60 leucocytes in the final hour of TNF- α incubation and fluorescence was detected (D). Each experiment was repeated at least three times. Letters indicate groups significantly different using the PLSD test with $P < 0.05$. In Figure 4C, lower-case letters compare the signal of P-AKT^{Ser473} and capital letters the signal of P-eNOS^{Ser1177}. Columns labelled with the same letter do not differ significantly. (E) Immunostaining of caesarean delivered placentas for AKT1, AKT2, and AKT3 localized these to trophoblast and endothelial cells (AKT1), trophoblast only (AKT2), or endothelial cells only (AKT3).

reduction in AKT-mediated eNOS Ser¹¹⁷⁷ phosphorylation. Previous studies show a strong correlation between phosphorylation of eNOS and NO production; indeed that AKT-mediated Ser¹¹⁷⁷ eNOS phosphorylation promotes NO production and that genetic or pharmacological blockade of this reduces NO production.^{25,28}

A further vasoprotective function of VEGF-A-induced NO production is the ability of NO to inhibit leucocyte rolling and adhesion in blood vessels which is mediated, at least in part, by NO action on leucocytes.³⁵ In our study, phospho-eNOS was reduced by pre-treatment of HUVECs with sFLT1, which reduced both AKT Ser⁴⁷³ and eNOS Ser¹¹⁷⁷ phosphorylation. A further

effect of sFLT1 treatment that would increase adhesion was the induction of surface ICAM1 and VCAM1. This indeed led to enhanced HL60 adhesion when cells were treated with sub-maximal doses of TNF- α . Consistent with our *in vitro* results, administration of VEGF inhibitors to healthy mice leads to elevated levels of cytokines that promote metastasis and angiogenesis upon tumour cell injection.³⁶ Such activated endothelium could then facilitate adhesion and egress of tumour cells from the vasculature. An analogy can be drawn between this situation and that of pre-eclampsia, where maternal endothelial perturbation leads to vasoconstriction, manifested as hypertension, increased vascular



permeability leading to oedema, and glomerular endotheliosis causing proteinuria.^{2,3}

vWF is a well-recognized marker of endothelial dysfunction and circulating levels are elevated in both pre-eclampsia and HELLP syndrome (haemolysis, elevated liver enzymes, and low platelet count).²² In our experiments, sFLT1 treatment induced little surface vWF, and low doses of TNF-α only stimulated a moderate increase. However, sFLT1 and TNF-α acted synergistically to significantly increase vWF surface expression. Thus, sFLT1 antagonizes the vaso-protective effect of VEGF-A on vascular endothelium.

We selected TNF-α as a representative pro-inflammatory cytokine secreted by the stressed placenta, but other factors, such as IL-1β or IL-6, may also act in synergy with sFLT1. Animals treated with sFLT1 alone do not develop severe pre-eclampsia and HELLP syndrome, i.e. no haemolysis or thrombocytopenia.⁵ This suggests that other placentally derived soluble factors act in concert with sFLT1 to cause endothelial dysfunction, resulting in severe pre-eclampsia and HELLP. It is notable that, compared with sFLT1 treatment alone, the combination of sENG and sFLT1 induced severe clinical signs of pre-eclampsia,

including HELLP syndrome and cerebral oedema, which resembles eclampsia.¹⁶ Pregnancy is a state of increased inflammatory response²⁶ and it thus seems that sFLT1 sensitization of maternal endothelium only leads to endothelial dysfunction in the presence of other pro-inflammatory factors, which are secreted by the placenta during pregnancy.

Several splice variants of sFLT1 have been identified; three of which are known to be up-regulated in placenta of women with pre-eclampsia and contribute to total sFLT1 production by cytotrophoblast-like cells *in vitro*.³⁷ In this study, we used recombinant protein encoded by a single splice variant of sFLT1, and it is thus possible that slightly different results could have been obtained with the other splice variants. Another potential limitation of this study is the use of HUVECs to study the effect of sFLT1 and TNF-α. However, since pre-eclampsia affects a variety of vascular beds, it is likely that characteristics common to multiple different endothelial cell types are important. So, while HUVECs are not microvascular cells, they do share many of the important characteristics exhibited by such cells isolated from numerous vascular beds.

In conclusion, our study demonstrates that antagonizing the auto-crine actions of endothelial VEGF-A and/or PIGF by pre-incubation with recombinant sFLT1, anti-FLT1, anti-KDR, anti-VEGF-A, SU5614, or knocking-down *FLT1* or *KDR* transcripts, renders the cells more sensitive to the effects of low doses of TNF-α. Each treatment ultimately led to reduction in AKT Ser⁴⁷³ and eNOS Ser¹¹⁷⁷ phosphorylation (and hence activity) and increased endothelial activation, as measured by increases in endothelial ICAM1, VCAM1, ET-1, vWF, and leucocyte adhesion. sFLT1 and TNF-α are both secreted by the human placenta in response to oxidative stress, and our data demonstrate a mechanism by which these factors combine to cause dysfunction of maternal endothelium.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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