Elevated Placental Soluble Vascular Endothelial Growth Factor Receptor-1 Inhibits Angiogenesis in Preeclampsia

Shakil Ahmad, Asif Ahmed

Abstract—Preeclampsia is an inflammatory disorder in which serum levels of vascular endothelial growth factor (VEGF) and its soluble receptor-1 (sVEGFR-1, also known as sFlt-1) are elevated. We hypothesize that VEGF and placenta growth factor (PIGF) are dysregulated in preeclampsia due to high levels of sVEGFR-1, which leads to impaired placental angiogenesis. Analysis of supernatants taken from preeclamptic placental villous explants showed a four-fold increase in sVEGFR-1 than normal pregnancies, suggesting that villous explants in vitro retain a hypoxia memory reflecting long-term fetal programming. The relative ratios of VEGF to sVEGFR-1 and PIGF to sVEGFR-1 released from explants decreased by 53% and 70%, respectively, in preeclampsia compared with normal pregnancies. Exposure of normal villous explants to hypoxia increased sVEGFR-1 release compared with tissue normoxia (P<0.001), as did stimulation with tumor necrosis factor-α (P<0.01). Conditioned medium (CM) from normal villous explants induced endothelial cell migration and in vitro tube formation, which were both attenuated by pre-incubation with exogenous sVEGFR-1 (P<0.001). In contrast, endothelial cells treated with preeclamptic CM showed substantially reduced angiogenesis compared with normal CM (P<0.001), which was not further decreased by the addition of exogenous sVEGFR-1, indicating a saturation of the soluble receptor. Removal of sVEGFR-1 by immunoprecipitation from preeclamptic CM significantly restored migration (P<0.001) and tube formation (P<0.001) to levels comparable to that induced by normal CM, demonstrating that elevated levels of sVEGFR-1 in preeclampsia are responsible for inhibiting angiogenesis. Our finding demonstrates the dysregulation of the VEGF/PIGF axis in preeclampsia and offers an entirely new therapeutic approach to its treatment. (Circ Res. 2004;95:884-891.)

Key Words: angiogenesis ■ sFlt-1 ■ VEGF ■ PLGF ■ preeclampsia

Hypertension in pregnancy predisposes women to cerebral hemorrhage. Anecdotal reports imply that there may also be an increased risk of myocardial infarctions developing in these women. Preeclampsia is a severe form of pregnancy-induced hypertension affecting 7% to 10% of all first-time pregnancies. The maternal syndrome is characterized by an elevated blood pressure, proteinuria, and edema. Recent studies showed that women with a history of preeclampsia have higher circulating concentrations of fasting insulin, lipid and coagulation factors postpartum, and that there is a specific defect in endothelial-dependent vascular function. The cause of preeclampsia remains unknown. However, the placenta is involved, because preeclampsia can occur in hydatidiform mole when placental tissue alone is present; the delivery of the placenta is the only known cure for preeclampsia. Insufficient adaptation of the decidual and intramyometrial portions of the spiral arterioles in preeclampsia results in reduced utero-placental blood flow, leading to local placental hypoxia. Vascular endothelial growth factor (VEGF) is upregulated by hypoxia and is a potent vascular-protective and angiogenic factor in the placenta. VEGF mediates its signal via two tyrosine kinase receptors, VEGF receptor-1 (VEGFR-1/Flt-1) and VEGFR-2 (KDR/Flk-1). VEGFR-1 can also be expressed as a soluble protein and is generated by alternative splicing of the fms-like tyrosine kinase (flt-1) gene. Soluble VEGFR-1 (sVEGFR-1/sFlt-1) mRNA is expressed at high levels in the placenta and is produced by villous and extravillous trophoblasts. Vuorela et al demonstrated that levels of the VEGF-binding protein in amniotic fluid were higher in women with preeclampsia compared with those with normal pregnancy, and this protein is increased in preeclamptic placenta. Soluble VEGFR-1 has strong antagonistic activity as it binds to all isoforms of VEGF and placenta growth factor (PIGF), and it can also form dominant-negative complexes with mitogenically competent full-length VEGF-2. More recent studies showed that serum levels of sVEGFR-1 in women with preeclampsia were elevated. In addition to the effect of sVEGFR-1 on the maternal circulation as reported by Maynard et al, we addressed whether sVEGFR-1 could represent a potential mechanism for poor

Original received March 25, 2003; resubmission received April 29, 2004; revised resubmission received September 27, 2004; accepted September 29, 2004.

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Circulation Research is available at http://www.circresaha.org

DOI: 10.1161/01.RES.0000147365.86159.f5

884
placental development in preeclampsia. The aim of this study was to test the hypothesis that placenta from pregnancies complicated with preeclampsia release higher levels of sVEGFR-1, which may impair angiogenesis, and this increase in sVEGFR-1 may be attributable to hypoxia and elevated cytokines.

Materials and Methods
Reagents
Recombinant VEGF<sub>165</sub> and sVEGFR-1 ectodomains (sVEGFR-1) were purchased from (RELIATech, Germany). Growth factor-reduced Matrigel was obtained from BD Biosciences (Cowley, UK). Polycarbonate filters (8-μm pore size, polycyvinylpyrrolidone-free) were from Receptor Technologies Ltd (Adderbury, UK). All other cell culture reagents and chemicals were obtained from Sigma Chemical Co Ltd (Poole, UK).

Cell Culture
Human umbilical vein endothelial cells (HUVECs) were isolated, characterized, and cultured as previously described. Experiments were performed on second- or third-passage HUVECs. Stably transfected porcine aortic endothelial (PAE) cells expressing human VEGFR-1 (PAE<sub>VEGFR-1</sub>) were obtained from Dr Johannes Waltenberger (Ulm, Germany). Full-length Flt-1 cDNA (clone 3 to 7) was cloned into the cytomegalovirus-based eukaryotic expression vector pcDNAI/Neo and transfected into PAE cells using electroporation as described before. All cell types were grown at 37°C, 5% CO<sub>2</sub> in a humidified incubator and routinely passaged when 90% confluent.

Tissue Collection
Human placental tissue were obtained from uncomplicated term pregnancies delivered by elective cesarean section for breech presentation or a recurring indication in otherwise uncomplicated pregnancies as previously described. In addition, similar gestation-matched placental tissues were collected by elective cesarean section from pregnancies complicated by preeclampsia or intrauterine growth restriction. Preeclampsia was defined as blood pressure elevation cytokines, growth factors, or control media were assayed in triplicate.

Western Blotting
Proteins were extracted from placental tissues and subjected to Western blot analysis as previously described. In brief, 50 μg total protein was separated on 8% SDS-PAGE and transferred to nitrocellulose membranes (Amersham-Pharmacia). Membranes were incubated with anti-VEGFR1 (Flt1/1/extracellular domain) antibody (1:1000) or anti-VEGFR1 (carboxy terminus) antibody (1:1000) (Autogen Bioclear), or anti-α-tubulin antibody (1:1000) at 4°C overnight. Antibody reactions were detected using the ECL detection kit (Amersham-Pharmacia).

Immunohistochemistry
Serial 3-μm sections of formalin-fixed, paraffin-embedded placental tissue were used for immunohistochemistry as previously described. Anti-VEGF (Autogen Bioclear) (1:250) and anti-VEGFR-1 extracellular domain (R&D Systems) (1:200) antibodies were used. The staining was analyzed using a Nikon inverted microscope and an Image Pro Plus image analysis software (Media Cybernetics).

Immunoprecipitation
Immunoprecipitation of sVEGFR-1 protein on CM from normal and preeclampic placentals explants was performed as previously described. Briefly, CM from normal and preeclamptic placentas explants was precleared by adding 50 μL of protein-A Sepharose beads (150 mg/mL; Sigma) for 2 hours at 4°C. The supernatant after centrifugation was collected and incubated with anti-VEGFR1 (Flt1/1/extracellular domain) antibody or nonimmune IgG overnight at 4°C with shaking. After incubation with primary antibody, 50 μL of protein-A beads was added and again incubated for 2 hours at 4°C with shaking. The mixture was centrifuged and the pellet discarded. Thereafter, in vitro tube formation and cell migration assays were performed on sVEGFR-1–depleted CM as described.

In Vitro Migration Assay
Chemoattrax of HUVEC was performed in a modified Boyden chamber as previously described. Briefly, placental explant CM was pretreated with sVEGFR-1 (100 ng/mL) for 30 minutes before stimulation. The filters were then fixed and stained with Diff-Quik (Harleco), and 10 fields at ×200 magnification were counted.

In Vitro Tube Formation Assay
In vitro formation of tubular structure was studied using PAE<sub>VEGFR-1</sub> cells on growth factor-reduced Matrigel diluted 1:1 in ice-cold DMEM as previously described. Briefly, placental explant CM was pretreated with VEGF (50 ng/mL) or sVEGFR-1 (100 ng/mL) for 30 minutes before stimulation. After incubation for 4 hours, cells were observed with a Nikon inverted microscope and experimental results recorded with an Image Pro Plus image analysis software (Media Cybernetics).

Statistical Analysis
All data are expressed as mean ± SEM. Statistical comparisons were performed using one-way ANOVA followed by the Student-Newman-Keuls test as appropriate. Statistical significance was set at a value of P < 0.05.

Results
VEGF-A but not PI GF Levels Are Increased in Preeclampsia
Placental villous explants were incubated in DMEM containing 0.2% BSA for 24 hours, and the CM was analyzed for levels of VEGF and PI GF using a sandwich ELISA (Figure 1). Preeclamptic placenta displayed a 2-fold increase in
VEGF release (mean ± SEM: 117 ± 3.7 ng/mL per mg; P < 0.001; n = 12) compared with normal placenta (55 ± 6.4 ng/mL per mg; n = 15), whereas placenta from fetal growth-restricted pregnancies showed 1.5-fold increase in release of VEGF (90 ± 10.8 ng/mL per mg; n = 9) (Figure 1A). In contrast, no significant change in PlGF levels was observed in preeclamptic and fetal growth-restricted placenta (Figure 1B).

**Preeclamptic Placentas Produce Elevated Levels of sVEGFR-1**

Because the activity of VEGF and PI GF is modulated by sVEGFR-1, we assayed levels of sVEGFR-1 from pregnancies complicated with preeclampsia and fetal growth restriction. The release of sVEGFR-1 from placental villous explants incubated for 24 hours was significantly higher in preeclamptic (mean ± SEM: 128 ± 9.8 ng/mL per mg; P < 0.001; n = 12) and fetal growth-restricted placenta (47 ± 4.1 ng/mL per mg; P < 0.01; n = 9) compared with normal-term placenta (28 ± 1.7 ng/mL per mg; n = 15) (Figure 2A).

To determine whether levels of VEGF and PI GF are altered, the ratios of sVEGFR-1 to PI GF and VEGF were calculated in normal and diseased placenta (Table). In preeclampsia, the relative ratio of VEGF to sVEGFR-1 and PI GF to sVEGFR-1 decreased by 53% and 70%, respectively, compared with normal pregnancies (Table). This decrease clearly shows that there is a net increase in sVEGFR-1 levels in preeclampsia and that elevated levels of VEGF are not sufficient to compensate for the inhibitory effect of sVEGFR-1.

To confirm the upregulation of sVEGFR-1, and to exclude the possibility of compensatory increases in full-length VEGF and PI GF, we assayed the ratios of sVEGFR-1, PI GF-1, and VEGF in normal and diseased placenta (Table). In preeclampsia, the relative ratio of VEGF to sVEGFR-1 and PI GF to sVEGFR-1 decreased by 53% and 70%, respectively, compared with normal pregnancies (Table). This decrease clearly shows that there is a net increase in sVEGFR-1 levels in preeclampsia and that elevated levels of VEGF are not sufficient to compensate for the inhibitory effect of sVEGFR-1.

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addition, it was proposed that placental angiogenesis is defective in preeclampsia. Because endothelial cell migration is an essential component of angiogenesis, we investigated whether the increase in sVEGFR-1 levels in preeclampsia is responsible for the compromised angiogenesis. Precipitation of exogenous sVEGFR-1 significantly attenuated HUVEC migration in response to VEGF (Figure 5A). Likewise, a significant decrease in migration was seen in CM from normal placental explants (54 ± 5.5 cells/field; P < 0.001) compared with CM from normal placental explants (152 ± 8.8 cells/field) (Figure 5B). Furthermore, to conclusively demonstrate that the inhibitory effect of the CM from preeclamptic placenta was solely attributable to the increased levels of sVEGFR-1, sVEGFR-1 was removed by immunoprecipitation from normal and preeclamptic CM and cell migration

### Ratios of VEGF and PI GF to sVEGFR-1 Are Decreased in Preeclampsia

<table>
<thead>
<tr>
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<th>VEGF/sVEGFR-1</th>
<th>PI GF/sVEGFR-1</th>
<th>VEGF/PI GF</th>
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<tr>
<td>Normal (n=15)</td>
<td>1.942</td>
<td>1.334</td>
<td>1.456</td>
</tr>
<tr>
<td>PE (n=12)</td>
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<td>0.396*</td>
<td>2.297*</td>
</tr>
<tr>
<td>FGR (n=9)</td>
<td>1.889</td>
<td>0.704</td>
<td>2.682</td>
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Levels of VEGF, PI GF, and sVEGFR-1 released from placental villous explants from normal pregnancies (Normal) and those complicated with preeclampsia (PE) or fetal growth restriction (FGR) were expressed as ratios. Data are ratios of mean of five separate experiments performed in triplicate.

**P<0.05 vs control.

### VEGF and Tumor Necrosis Factor-α Induce the Release of sVEGFR-1

Because VEGF and tumor necrosis factor (TNF)-α levels are reported to be elevated in pregnancies complicated by preeclampsia, we therefore investigated the effect of exogenous VEGF and TNF-α on sVEGFR-1 release. Both VEGF and TNF-α induced a concentration-dependent release of sVEGFR-1 into the CM from normal placental villous explants (Figure 3A and 3B).

### Hypoxia Stimulates Release of sVEGFR-1 in Normal Placenta

In contrast to VEGF-2, VEGF-1 is upregulated by hypoxia. This is mediated by HIF-1α binding to a hypoxia response element in the flt-1 gene promoter. We therefore determined the effect of hypoxia on sVEGFR-1 release from normal placental explants (Figure 4). As expected, exposure of villous explants to 1% O2 (hypoxia) significantly increased the release of sVEGFR-1 (45.1 ± 1.4 ng/mL per mg; P < 0.001) compared with the release at 5% O2 (22.0 ± 1.7 ng/mL per mg), which approximates to levels in the placenta.

### Soluble VEGFR-1 in Preeclamptic Placental CM Inhibits Cell Migration

VEGF and PI GF are differentially expressed during gestation and are important regulators of placental development. In addition, it was proposed that placental angiogenesis is
were reassessed. Conditioned media from preeclamptic placental villous explants depleted of sVEGFR-1 significantly restored endothelial cell migration (138±6.3 cells/field; \( P<0.001 \)) to levels similar to that observed with normal CM (148±7.7 cells/field; \( n=5 \)) (Figure 5B).

Addition of increasing concentrations of sVEGFR-1 (1 ng/mL to 200 ng/mL) to preeclamptic CM did not further reduce endothelial cell migration, demonstrating that preeclamptic CM contains saturating concentrations of the soluble receptor (Figure 5C). In contrast, addition of increasing doses of exogenous sVEGFR-1 to CM from normal placental explants significantly attenuated endothelial cell migration in a dose-dependent manner (Figure 5C). Total inhibition of cell migration was achieved between 100 and 200 ng/mL (45±3.1 cells/field), which was of the same order of magnitude as the release of sVEGFR-1 from preeclamptic placenta. In addition, supplementation of exogenous VEGF (50 ng/mL) to the preeclamptic CM (142±6.1 cells/field) abolished its inhibitory effect on endothelial cell migration (Figure 5D) to levels similar to CM from normal placenta (138±5.1 cells/field), suggesting that there was a net defect in VEGF activity.

Soluble VEGFR-1 in Preeclamptic Placental CM Inhibits In Vitro Tube Formation

We have previously shown that VEGF induces tube formation via VEGFR-1 when plated on growth factor-reduced Matrigel.14 Addition of CM from normal placental villous explants to PAE-VEGFR-1 cells produced complete tubular structures (Figure 6AII). Conditioned media from preeclamptic placental villous explants also induced in vitro tube formation; however, PAE-VEGFR-1 cells formed an incomplete and narrow tubular network that remained poorly developed (Figure 6AIII). In contrast to PAE-VEGFR-1 cells, the PAE-VEGFR-2 and PAEWT cells were unable to establish a network of tubular-like structures on Matrigel under basal conditions or when stimulated with placental villous explants CM (data not shown), demonstrating that the effect observed was attributable to VEGFR-1 under these conditions.

Quantitative analysis showed a significant increase in total tube length when endothelial cells were stimulated with CM from normal placental explants (total tube length, 10672±46.5 \( \mu \)m/field; \( P<0.001 \)) compared with preeclamptic placenta (7836±59.4 \( \mu \)m/field; \( P<0.05 \)) (Figure 6B). Pre-incubation of CM from normal explants with sVEGFR-1 caused a significant reduction in tubular networks (4121±154.5 \( \mu \)m/field; \( P<0.001 \)). However, pre-incubation of CM from preeclamptic explants with exogenous sVEGFR-1 had no significant effect on tube formation.

CM from preeclamptic placental explants depleted of sVEGFR-1 significantly restored in vitro angiogenesis (Figure 6C). Total tube length increased from 7836±59.4 \( \mu \)m/field to 11 934±378.3 \( \mu \)m/field (\( P<0.001 \)) and was comparable to the total tube length induced by untreated CM from normal placental villous explants (12 406±113.3 \( \mu \)m/field) (Figure 6C). Furthermore, addition of exogenous VEGF (50 ng/mL) to CM from preeclamptic placenta also increased tube formation from 4927±420.6 \( \mu \)m/field to 9242±374 \( \mu \)m/field (\( P<0.001 \)) (Figure 6D), whereas immunoprecipitation of sVEGFR-1 or addition of VEGF to CM from normal placental villous explants depleted of sVEGFR-1 did not further reduce endothelial cell migration.
placental explants had no significant effect on in vitro angiogenesis (Figure 6C and 6D).

**Discussion**

Recently, an in vivo animal model demonstrated that overexpression of sVEGFR-1 leads to hypertension, proteinuria, and glomerular endotheliosis, conditions that are similar to preeclampsia. In addition to the effect of sVEGFR-1 on the maternal circulation as reported by Maynard et al., we addressed whether the elevated sVEGFR-1 in patients with preeclampsia has an anti-angiogenic effect during placental development. The data presented here show that preeclamptic placenta produce high levels of sVEGFR-1. CM from pre-eclamptic placenta attenuated endothelial cell migration and in vitro tube formation, two key markers of angiogenesis, indicating that raised levels of sVEGFR-1 in placenta may explain the poorly developed feto-placental vasculature associated with this disorder. Although normal placenta CM promoted angiogenesis, pre-incubation of CM with exogenous sVEGFR-1 significantly attenuated endothelial cell migration and tube formation, whereas addition of exogenous sVEGFR-1 to preeclamptic CM did not further inhibit angiogenesis, attributable to saturating concentration of this soluble receptor. Removal of sVEGFR-1 by immunoprecipitation from preeclamptic CM significantly restored migration and tube formation to levels that were similar to normal CM. Thus, VEGF is unlikely to be coprecipitated with sVEGFR-1, because there is sufficient VEGF activity to fully restore angiogenesis, which suggests that the elevated level of sVEGFR-1 in preeclampsia is specifically responsible for inhibiting placental angiogenesis.

The increase in plasma VEGF and PIGF levels observed in normal pregnancies is significantly attenuated in pregnancies complicated by preeclampsia. Both of these growth factors are vascular-protective. PIGF upregulates Bcl-2 expression and sustains capillary-like tube networks over many days of primary microvascular endothelial cells grown on collagen gels. In the present study, the ratios of VEGF to sVEGFR-1 and PIGF to sVEGFR-1 were significantly lower in preeclampsia. Despite the fact that mice lacking plgf gene are viable, our study implies that the VEGF/PIGF axis is dysregulated in preeclampsia, and further suggests that PIGF is an important factor for normal pregnancy in women. Because the placenta vasculature is “immature” in preeclampsia, soluble VEGFR-1 acting as a sink to reduce the free levels of VEGF and PIGF in preeclampsia may result in loss of endothelial cell integrity and increased cellular apoptosis. Preeclampsia is associated with increased trophoblast apoptosis and altered placental vascular reactivity.

The elevated level of sVEGFR-1 detected in placenta from women with preeclampsia is probably attributable to placental hypoxia resulting from utero-placental insufficiency. Defective remodeling of the endometrial spiral arteries is the most widely recognized predisposing factor for preeclampsia. As a result, perfusion of the intervillous space is impaired, leading to placental hypoxia. The finding that VEGF-mediated trophoblast migration was blocked by sVEGFR-1 suggests that sVEGFR-1 may modulate VEGF activity in utero-placental remodeling. Consistent with these
increased circulating levels of TNF-α in preeclampsia. There is an increased circulating levels of TNF-α. Furthermore, intermittent perfusion of the placenta, secondary to reduced trophoblast invasion, causes increased secretion of TNF-α.

The present study shows that TNF-α, in a concentration-dependent manner, stimulates the release of sVEGFR-1 from placental explants. Because the levels of sVEGFR-1 production in hypoxia or on stimulation with TNF-α resulted in a relatively smaller increase in sVEGFR-1, it is likely that these stimuli act synergistically to potentiate the release of sVEGFR-1 in vivo. Other cytokines also may be involved in inducing the release of sVEGFR-1. This would suggest that increased cytokine production in women with preeclampsia induces the release of sVEGFR-1, which in the placenta inhibits angiogenesis and also has a deleterious effect on the maternal vascular endothelium.

 Likewise, in preeclampsia, but not in normal pregnancies, there is activation of neutrophils and monocytes during the utero-placental passage. On activation, leukocytes release their granular contents, which are capable of mediating vascular damage. Soluble VEGFR-1 may be one such culprit in this process. The addition of exogenous VEGF can induce the release of sVEGFR-1 from cultured endothelial cells in a concentration-dependent manner, and human hematopoietic cell lines also produce sVEGFR-1. Thus, the raised level of VEGF in the maternal circulation may contribute to the increased level of sVEGFR-1 by stimulating release of sVEGFR-1 from the maternal endothelium and leukocytes.

In normal pregnancy, the rapid growth of placenta and the associated vascularization occurs from the second trimester of pregnancy onward. In preeclampsia, during this period, circulating levels of sVEGFR-1 are elevated. Preeclamptic placental explants released ≈140 ng/mL per milligram of sVEGFR-1, which was of the same order of magnitude as exogenous sVEGFR-1 required to inhibit angiogenesis. The loss of >70% of the PI GF activity in preeclampsia strongly supports our premise that the VEGF/PI GF axis is dysregulated. Furthermore, the high expression of sVEGFR-1 in preeclampsia may form dominant-negative complexes with full-length VEGF-2 to inhibit angiogenesis much earlier in pregnancy. The fact that removal of sVEGFR-1 by immunoprecipitation from preeclamptic CM significantly restored angiogenesis further suggests that the elevated level of sVEGFR-1 in preeclampsia is likely to be responsible for the poorly developed feto-placental vasculature associated with this disorder. These findings provide potential therapeutic approaches for the prevention and treatment of preeclampsia and suggest that pharmacological intervention to inhibit sVEGFR-1 may be worthy of investigation.

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Circ Res. 2004;95:884-891; originally published online October 7, 2004;
doi: 10.1161/01.RES.0000147365.86159.f5
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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