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Thrombin Regulates Soluble fms-Like Tyrosine Kinase-1 (sFlt-1) Expression in First Trimester Decidua

Implications for Preeclampsia

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The primary placental defect in preeclampsia is shallow trophoblast invasion of the decidua leading to incomplete vascular transformation and inadequate uteroplacental perfusion. Soluble fms-like tyrosine kinase-1 (sFlt-1) seems to interfere with these events by inhibiting local angiogenesis and/or by impeding trophoblast invasion. Preeclampsia is also associated with maternal thrombophilias and decidual hemorrhage, which form thrombin from decidual cell-expressed tissue factor. Although sFlt-1 is highly expressed by trophoblasts, sFlt-1 expression has not been studied in decidual cells, which are the predominant cell type encountered by invading trophoblasts. Here, we demonstrate that isolated decidual cells express sFlt-1 mRNA, suggesting that they can synthesize sFlt-1. Moreover, in first trimester decidual cells, thrombin enhanced sFlt-1 mRNA levels, as measured by quantitative reverse transcriptase-polymerase chain reaction, and levels of secreted sFlt-1 protein, as measured by enzyme-linked immunosorbent assay. The thrombin antagonist hirudin blocked this effect, demonstrating that active thrombin is required. Emphasizing the specificity of the thrombin response, neither interleukin-1 β nor tumor necrosis factor- α affected sFlt-1 expression in the decidual cells. In contrast to first trimester decidual cells, thrombin did not affect sFlt-1 levels in cultured term decidual cells. In early pregnancy, thrombin may act as an autocrine/paracrine enhancer of sFlt-1

expression by decidual cells to promote preeclampsia by interfering with local vascular transformation. (Am J Pathol 2007, 170:1398–1405; DOI: 10.2353/ajpath.2007.060465)

Implanting human cytotrophoblasts invade the decidua and then breach decidual arteries and arterioles to replace the smooth muscle tunica media and endothelium.¹ During this process, the trophoblast epithelial cell adhesion molecule phenotype is converted to an endothelial cell-like adhesion molecule phenotype. This transformation, termed pseudovasculogenesis,² is implicated in the enhanced vascular conductance and blood flow to the intervillous space required for growth and development of the fetoplacental unit.^{3,4}

Impaired trophoblast invasion is the primary placental defect of preeclampsia and fetal growth restriction.⁴ Shallow decidual invasion leads to incomplete vascular conversion and failed pseudovasculogenesis.² The resulting uteroplacental ischemia leads to hypoxia and a blood supply that can be inadequate to meet the demands of the growing fetoplacental unit.⁵ Preeclamptic placentae contain elevated levels of hypoxia-inducible transcription factors.^{6,7} Hypoxic conditions enhance expression of the potent angiogenic molecules, vascular endothelial cell growth factor (VEGF) and placenta growth factor (PlGF) by human cytotrophoblasts *in vivo* and *in vitro*.^{8,9} Paradoxically, despite its association with placental hypoxia, preeclampsia is associated

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with reduced circulating levels of VEGF and PlGF.^{10–12} These changes are likely due to increased levels of the angiogenic inhibitor, soluble fms-like tyrosine kinase-1 (sFlt-1), a splice variant of the VEGF receptor Flt-1. Elevated plasma levels of sFlt-1 have been found as early as 5 weeks before the clinical manifestation of preeclampsia.¹³

The dependence of uteroplacental blood flow on trophoblast-mediated remodeling of decidual blood vessels taken together with the involvement of angiogenesis in vascular remodeling¹⁴ has focused attention on VEGF and PlGF in establishing the fetoplacental circulation. These factors act via specific Flt-1 and KDR surface receptors. They are inhibited by binding to sFlt-1, which is highly expressed by normal cytotrophoblasts¹⁵ and further elevated in cytotrophoblasts from preeclamptic placentae.¹⁶ The observation that VEGF transforms hematopoietic stem cells to endothelial cells¹⁷ suggests a mechanism by which inhibition of VEGF by excess sFlt-1 could block trophoblast-induced vascular transformation.

To enhance understanding of the physiological and pathological angiogenic milieu encountered by invading trophoblasts, we evaluated sFlt-1 expression by first trimester decidual cells, the predominant cell type at the implantation site.¹⁸ The effects of tumor necrosis factor- α (TNF α) and interleukin-1 β (IL-1 β) were assessed on sFlt-1 expression, since these proinflammatory cytokines are implicated in the early pathogenesis of preeclampsia including inhibition of trophoblast invasion of the decidua.^{19–22} Preeclampsia is also associated with underlying decidual hemorrhage,^{23–26} which generates thrombin when circulating factor VII binds to decidual cell-expressed tissue factor.^{27,28} Therefore, the effects of this hemostatic factor were also evaluated on sFlt-1 expression in the decidual cell monolayers. To determine the potential for progesterone to confer protection against TNF α , IL-1 β , and thrombin effects on sFlt-1 expression, decidual cells were incubated with each molecule added with estradiol (E₂) alone or with E₂ plus the progesterone medroxyprogesterone acetate (MPA).

Materials and Methods

Tissues

For first trimester, decidual specimens from nine elective terminations between 6 and 12 weeks of gestation were obtained with patient consent and under the approval of the Institutional Review Board of Bellevue Hospital. Placentas and attached fetal membranes were obtained at term from six nonlaboring patients with uncomplicated pregnancies undergoing repeat Cesarean deliveries at Yale-New Haven Hospital under Human Investigation Committee approval after receiving written informed consent. A small portion of each specimen was formalin-fixed and paraffin-embedded and then examined histologically for signs of underlying acute and chronic inflammation. The remainder of each specimen was used to isolate decidual cells.

Fibrin Staining

First trimester human placentas ($n = 6$) were obtained from patients undergoing elective termination of pregnancy by vacuum aspiration at 6 to 10 weeks of gestation. Approval for this study was granted by the Human Institutional Investigation Committee of the University of Siena. Informed consent was obtained from all women. Tissues were fixed in 10% buffered neutral formalin and embedded in paraffin. Slides of each specimen were stained with hematoxylin-eosin and histologically examined by a pathologist (P.T.). Only tissues from uncomplicated pregnancy were included in this study. Fibrin-specific Picro-Mallory staining was performed using a commercially available kit (Bio-Optica, Milan, Italy).

First Trimester Decidual Cell Cultures

Tissues were minced and digested with 0.1% collagenase type IV, as well as 0.01% DNase in cRPMI [RPMI + 10% fetal calf serum (FCS), 20 μ g/ml penicillin/streptomycin, and 1 μ l/ml Fungizone] (Sigma-Aldrich, St. Louis, MO) in a 37°C shaking water bath for 30 minutes. After washing with sterile phosphate-buffered saline (PBS), the digestate was filtered through 100-, 60-, and 40- μ m Millipore filters (Millipore Corporation, Billerica, MA), respectively. Cells were resuspended in cRPMI, seeded on polystyrene tissue culture dishes, and then grown to confluence in a standard 95% air/5% CO₂ incubator at 37°C and passaged. Cell aliquots were then frozen in 10% FCS/dimethyl sulfoxide (9:1) (Sigma-Aldrich) and stored in liquid nitrogen. Consistent with published results of stromal cells isolated from cycling endometrium, decidual cells isolated from first trimester were vimentin-positive and cytokeratin-negative. These cells display characteristic decidualization-related morphological changes and increased secretion of tissue factor, plasminogen activator inhibitor-1, and prolactin in response to progesterone.^{28,29}

Term Decidual Cell Cultures

The decidua was scraped from the maternal surface of the chorion, minced, and digested in Ham's F-10 + 10% charcoal-stripped calf serum (SCS) (Flow Laboratories, Rockville, MD) containing 2.5 mg/ml collagenase (200 U/mg) (Worthington Biochemical Corp, Freehold, NJ) in a shaking water bath at 37°C for 30 minutes, suspended as 1 g of tissue per 10 ml of solution. After adding 6.25 U of DNase (Sigma-Aldrich) per milliliter of digestate, the incubation was continued for another 45 minutes. The final digestate was passed through a 23-gauge needle five times to dissociate remaining cell clusters. The isolated cells were centrifuged at 1500 rpm for 5 minutes at 4°C and then washed in Ham's F-10. This procedure was repeated three times, and the final cell pellet was resuspended (1 g of tissue/ml) in 20% Percoll (Sigma-Aldrich), layered on a (60%:50%:40%) discontinuous Percoll gradient, and then centrifuged at 22,000 rpm for 20 minutes at 4°C. The top cell layer was collected, washed, resus-

pended in Ham's F-10 without serum, and centrifuged at 1500 rpm for 5 minutes at 4°C. After repeating this procedure, the resulting cell pellet was resuspended in 40% Percoll (1 g of tissue/ml), layered on a discontinuous (55%:50%:40%) Percoll gradient, and then centrifuged at 22,000 rpm for 20 minutes at 4°C. The top cell layer was washed twice in serum-free Ham's F-10 and then centrifuged at 1500 rpm for 5 minutes at 4°C. The cell pellet was resuspended in Ham's F-10 + 10% SCS, and decidual cells were counted in a hemocytometer. Trypan blue exclusion identified >95% of the isolated decidual cells as viable. The cultures were grown to confluence in a standard 95% air/5% CO₂ incubator at 37°C and passaged.

To determine whether stromal/decidual cells derived from the decidua basalis behave in a similar manner as those isolated from the decidua parietalis, the basalis was isolated from the maternal side of the placenta in one normal, uncomplicated term pregnancy. Subsequent processing and culturing was performed (in duplicate) as described above.

Experimental Incubations

Decidual cells were seeded onto polystyrene tissue culture-treated flasks and incubated in basal medium, a phenol red-free 1:1 (v/v) mix of Dulbecco's modified Eagle's medium (Sigma-Aldrich) and Ham's F-12 (Flow Laboratories), with 1% antibiotic-antimycotic (Invitrogen, Carlsbad, CA) supplemented with 10% SCS. Both first trimester and term decidual cells were harvested using trypsin/ethylenediamine tetraacetic acid and analyzed by flow cytometry with anti-CD45 and anti-CD14 monoclonal antibodies (BD PharMingen, San Diego, CA) to monitor the presence of leukocytes after each passage. Only cell cultures found to be leukocyte-free (<1%) were used for subsequent experimental incubations. The cultured cells were all vimentin-positive and cytokeratin-negative on immunohistochemistry.

Confluent cultures were incubated in parallel in basal medium containing either 10⁻⁸ mol/L E₂ or E₂ + 10⁻⁷ mol/L MPA (Sigma-Aldrich). MPA was used in these experiments in place of native progesterone, which is rapidly metabolized *in vitro*.³⁰ After 7 days, the cultures were washed twice with PBS to remove residual serum. The cultures were then switched to a defined medium (DM) consisting of basal medium plus ITS⁺ (Collaborative Research, Waltham, MA), 5 μmol/L FeSO₄, 0.5 μmol/L ZnSO₄, 1 nmol/L CuSO₄, 50 μg/ml ascorbic acid (Sigma-Aldrich), and 50 ng/ml epidermal growth factor (Becton-Dickinson, Bedford, MA) with the corresponding steroids ± IL-1β or TNFα or (R&D Systems, Minneapolis, MN) or thrombin (American Diagnostica, Greenwich, CT). In co-incubations with thrombin and hirudin (Sigma-Aldrich), a 1:1 mixture of these compounds were preincubated for 30 minutes at room temperature before exposure to cultured decidual cells.

After the experimental test period, first trimester and term decidual cells were harvested in ice-cold PBS using a cell scraper and then pelleted. Cell proteins were ex-

tracted in an ice-cold lysis buffer of Tris-buffered saline with 1% Triton X-100, 1 mmol/L phenylmethanesulfonyl fluoride (Sigma-Aldrich), and Complete protease inhibitor cocktail (Roche, Mannheim, Germany) and then sonicated. Cell lysates and conditioned medium supernatants were stored at -70°C. Parallel incubations of cultured decidual cells were run for RNA analysis.

Laser Capture Microdissection

To perform laser capture microdissection (LCM), serial 4-μm cryostat sections of term placentas/fetal membranes were mounted on uncoated glass slides, fixed in 70% ethanol, and stained with the HistoGene LCM frozen section staining kit (Arcturus, Mountain View, CA). Decidual cells were isolated using a PixCell II LCM system equipped with an Olympus microscope (Arcturus). Captured microdissected decidual cells from two different slides were pooled for subsequent analyses.

Biochemical Assays

The total cell protein content in the cell lysates was determined using a modified Lowry assay (Bio-Rad Laboratories, Hercules, CA). Commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems) were used to measure immunoreactive levels of sFlt-1, VEGF, and PlGF in the cell-conditioned medium according to instructions provided by the manufacturer. The sFlt-1 ELISA assay has a sensitivity of 5.0 pg/ml, and intra-assay and interassay coefficients of variation are 3.3 and 7.6%, respectively. The VEGF ELISA assay has a sensitivity of 5.0 pg/ml, and intra-assay and interassay coefficients of variation are 4.7 and 6.7%, respectively. The PlGF ELISA assay has a sensitivity of 7.0 pg/ml, and intra-assay and interassay coefficients of variation are 6.1 and 9.5%, respectively. ELISA results were normalized to total cell protein content.

RT-PCR and Real-Time Quantitative RT-PCR

Total RNA from cultured cells was extracted with Tri Reagent (Sigma-Aldrich). Total RNA from the LCM-captured cells was extracted using the RNeasy micro kit (Qiagen Inc., Valencia, CA) according to the manufacturer's recommendations. This kit uses a procedure that includes digestion with an RNase-free DNase to remove contaminant DNA. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with a kit from Invitrogen on an Eppendorf Mastercycler (Eppendorf, Westbury, NY). For each RNA specimen, a negative control was prepared by omitting the reverse transcriptase. Characteristics of the PCR products were confirmed by direct sequence analysis.

The sFlt-1 mRNA on LCM-captured cells was detected by RT-PCR. In brief, total RNA was diluted in 10 mmol/L Tris-HCl, 50 mmol/L KCl, and 5 mmol/L MgCl₂, pH 8.3, containing 50 U of Moloney murine leukemia virus reverse transcriptase, 20 U of placental RNase inhibitor, deoxy-NTPs (dNTPs; 1 mmol/L each), 2.5 μmol/L oligo d(T) primers (Invitrogen) in 20 μl of volume. The mixture

was incubated at 42°C for 60 minutes, 99°C for 5 minutes, and 5°C for 5 minutes in a programmable thermal cycler (Bio-Rad). For each RNA specimen, a negative control was prepared by omitting the reverse transcriptase. Two microliters of RT reaction product were added to a mix containing 5× reaction buffer [300 mmol/L Tris-HCl, 75 mmol/L (NH₄)₂SO₄, and 7.5 mmol/L MgCl₂, pH 8.5], dNTP mixture (final concentration 0.25 mmol/L), 1.0 U of cloned *Thermus aquaticus* DNA polymerase (Invitrogen), and sFlt-1 primers (final concentration 0.4 μmol/L) in a volume of 50 μl. PCR was performed in a programmable thermal cycler (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as housekeeping gene. Primer sequences for G3PDH were: forward 5'-GAAGGTGAAGGTCGGAGTC-3', and the reverse was 5'-GAAGATGGTGTATGGGATTTTC-3' with a product size of 226 bp. Primer sequences for sFlt-1 were the same as those used for the quantitative real-time PCR (shown below). Amplifications were performed for 1 minute at 94°C, 1 minute at 58°C, and 1 minute at 72°C for 45 cycles followed by a final 10 minutes at 72°C. For each specimen, a blank was prepared using 2 μl of the corresponding RT blank. One-fifth of each PCR solution was fractionated by electrophoresis in a 1.8% agarose gel. Gels were stained with ethidium bromide, destained, and photographed.

Figure 1. Fibrin deposition in first trimester placenta and decidua. Conspicuous deposition of fibrin stained in red by the Picro-Mallory reagent indicated by **arrows** (original magnification, ×200). Fibrin staining is noted (**A**) in extravillous cytotrophoblasts invading the decidua (**D**, decidua; **T**, trophoblasts), and adjacent to anchoring villus extravillous cytotrophoblasts (**B**).

To perform quantitative real-time RT-PCR, reverse transcription was initially performed with Superscript II reverse transcriptase (Invitrogen). A standard curve was created between 40 pg to 2.5 ng of cDNA with a Roche Light Cycler by monitoring increasing fluorescence of PCR products during amplification. On establishing the standard curve, quantitation of the unknowns was determined with the Roche Light Cycler and adjusted to the quantitative expression of β-actin from the corresponding unknowns. Melting-curve analysis determined the specificity of the amplified products and the absence of primer-dimer formation. All products obtained yielded correct melting temperatures. The following primers were synthesized and gel-purified at the Yale DNA Synthesis Laboratory, Critical Technologies: β-actin, forward 5'-CGTACCACTGGCATCGTGAT-3' and reverse 5'-GTGTTGGCGTACAGGTCTTTG-3' (452 bp); sFLT-1, forward 5'-ACAACTCAGAGGTGAGCACTGCAA-3' and reverse 5'-TCCGAGCCTGAAAGTTAGCAA-3' (180 bp).

Statistical Analysis

Comparisons of control and the various treatment groups were performed using the Kruskal-Wallis analysis of variance on ranks test followed by the Student-Newman-Keuls posthoc test with *P* < 0.05 representing statistical significance.

Results

Picro-Mallory Staining of Implantation Sites

To provide indirect evidence of thrombin generation during implantation and placentation, we assessed levels of fibrin generation at first trimester implantation sites. Figure 1 demonstrates prominent staining for fibrin on extravillous cytotrophoblasts invading the decidua (Figure 1A) and anchoring extravillous cytotrophoblasts (Figure 1B).

Figure 2. Reverse transcriptase-PCR analysis of sFlt-1 mRNA levels in micro-dissected decidual tissues. Total RNA of two decidua specimens (**lanes 1 and 3**) was reverse transcribed and amplified in the presence of sFlt-1 or G3PDH primers. For each specimen, a negative control, prepared by omitting the reverse transcriptase, was amplified and loaded onto the gel (**lanes 2 and 4**). Placental RNA was used as a positive control (**PC**). Forty-five cycles were run for each PCR. Characteristics of the PCR products were confirmed by direct sequence analysis. The size of the molecular weight makers (**lane M**; bp) is indicated.

Figure 3. Effects of E_2 , MPA and thrombin (Th) on sFlt-1 output by first trimester decidual cell monolayers. Confluent passaged, leukocyte-free decidual cells were incubated for 7 days in E_2 or E_2 + MPA and then switched to DM with corresponding steroid(s) $\pm$ 2.5 U/ml thrombin for 24 hours. Levels of sFlt-1 were measured by ELISA in conditioned DM and normalized to cell protein (details in Materials and Methods; $n = 9$, mean $\pm$ SEM). *Versus E_2 or E_2 + MPA; $P < 0.05$.

Figure 5. Effects of thrombin on sFlt-1 mRNA levels in E_2 and E_2 + MPA-treated first trimester decidual cell monolayers. Confluent decidual cells were primed for 7 days with E_2 or with E_2 + MPA and then switched to DM with corresponding steroid(s) $\pm$ thrombin (2.5 U/ml) for 5 hours (details in Materials and Methods; $n = 6$, mean $\pm$ SEM). Ordinate: sFlt-1 mRNA/b actin mRNA. *Versus E_2 or E_2 + MPA; $P < 0.05$.

Presence of sFlt-1 mRNA in the Decidua

That decidual cells are the predominant cell type of the peri-implantational decidua and are in close proximity to invading trophoblast prompted assessment of sFlt-1 expression by the decidua *in vivo*. Figure 2 displays sFlt-1 mRNA expression by decidual cells that were isolated by laser capture microdissection from uncomplicated pregnancies. A band corresponding to the sFlt-1 mRNA product is evident, suggesting that decidual cells have the capacity to synthesize sFlt-1 *de novo*.

Regulation of sFlt-1 and VEGF Expression in Cultured Decidual Cells

Because circulating levels of E_2 and progesterone are high following implantation, E_2 was used as the control incubation for evaluating the effects of the progestin MPA. In the first trimester, leukocyte-free, decidual cell monolayers Figure 3 indicates no significant differences in secreted sFlt-1 levels in parallel incubations with E_2 (0.22 ± 0.03 pg/ml/ μ g protein $\pm$ SEM) or with E_2 + MPA (0.26 ± 0.04). However, the addition of thrombin (2.5 U/ml) elicited statistically significant increases ($P < 0.05$) in sFlt-1 output in cultures incubated with either E_2 (7.3 ± 1.7 -fold; mean $\pm$ SEM) or with E_2 plus MPA (7.2 ± 1.9 -fold). Figure 4 indicates that hirudin acted as a pure thrombin antagonist in cultures incubated with either E_2 or with E_2 plus MPA. Thus, hirudin did not affect sFlt-1

Figure 4. Effects of hirudin on thrombin-enhanced sFlt-1 output by E_2 or E_2 + MPA-treated first trimester decidual cell monolayers. Confluent decidual cells primed for 7 days with E_2 or E_2 + MPA, and then switched to DM with corresponding steroid(s) $\pm$ thrombin, or hirudin, or thrombin + hirudin for 24 hours. Levels of sFlt-1 were measured by ELISA in conditioned DM and normalized to cell protein (details in Materials and Methods; $n = 2$, mean $\pm$ SD).

output when added alone, but it eliminated virtually all of the thrombin-mediated increase of sFlt-1 levels in the conditioned DM. Figure 5 confirms that steady-state sFlt-1 mRNA levels correspond to changes in secreted sFlt-1 protein. Accordingly, 2.5 U/ml thrombin augmented sFlt-1 mRNA levels by about sevenfold in decidual cell cultures incubated with either E_2 or with E_2 plus MPA.

Because circulating levels of E_2 and progesterone are high during the first trimester, additional effects of thrombin were assessed in decidual cell cultures primed with, and then maintained in, E_2 plus MPA. Figure 6 demonstrates that thrombin added from 0.025 to 2.5 U/ml elicited concentration-dependent increases in secreted levels of sFlt-1. Figure 7 underscores the specificity of the response to thrombin. Thus, compared with the more than sevenfold up-regulation of secreted sFlt-1 levels elicited by 2.5 U/ml thrombin, neither IL-1 β nor TNF α added at 10 ng/ml significantly altered levels of sFlt-1 in first trimester decidual cell-conditioned DM. In term decidual cells, neither thrombin nor IL-1 β nor TNF α significantly affected sFlt-1 output. As was the case with term decidual cells derived from the parietalis, term decidual cells derived from the basalis demonstrated a similar lack of response to thrombin, IL-1 β , or TNF α in sFlt-1 production (results not shown). The differential response to thrombin illustrates the gestational age dependence of this effect.

Figure 6. Concentration-dependent effects of thrombin on sFlt-1 output by E_2 + MPA-treated first trimester decidual cell monolayers. Confluent decidual cells were incubated in E_2 + MPA for 7 days and then switched to DM with E_2 + MPA alone or with thrombin at the concentrations indicated on the abscissa (from 0.025 to 2.5 U/ml) for 24 hours. Levels of sFlt-1 were measured by ELISA in conditioned DM and normalized to cell protein (mean $\pm$ SEM, $n = 3$). *Versus E_2 + MPA, $P < 0.05$; **versus + thrombin (0.25 U/ml), $P < 0.05$.

Figure 7. Effects of thrombin, TNF α , and IL-1 β on sFlt-1 output by E₂ + MPA-treated first trimester and term decidual cell monolayers. Confluent decidual cells were incubated for 7 days in E₂ + MPA and then switched to DM with corresponding steroids alone or with 2.5 U/ml thrombin, or 10 ng/ml TNF α or IL-1 β for 24 hours. Levels of sFlt-1 were measured by ELISA in conditioned DM and normalized to cell protein (details in Materials and Methods). For first trimester $n = 9$; for term $n = 6$; mean $\pm$ SEM. *Versus E₂ + MPA; $P < 0.05$.

Figure 8 compares the relative effects of thrombin on sFlt-1 versus VEGF production in first trimester decidual cell cultures. It demonstrates that thrombin selectively up-regulates sFlt-1 but not VEGF in first trimester decidual cells. In contrast with VEGF, secreted PIGF was not detectable (results not shown) in decidual cells under basal conditions or in response to thrombin, TNF α , or IL-1 β , suggesting an extradecidual source of PIGF during pregnancy.

Discussion

In 1989, Roberts et al³¹ hypothesized that the ischemic human placenta secretes circulating soluble factor(s) responsible for the widespread endothelial cell dysfunction characterizing the maternal syndrome of preeclampsia. Abundant evidence points to sFlt-1 as one such factor. Adenoviral transfer of the sFlt-1 gene induces preeclamptic symptoms in rats.³² In mice, loss of a single VEGF allele induced preeclamptic symptoms,³³ suggesting that sFlt-1 promotes endothelial cell dysfunction by inactivating VEGF. In support of this hypothesis, preeclamptic serum displays antiangiogenic activity that is

Figure 8. Effects of thrombin, TNF α , and IL-1 β on sFlt-1 versus VEGF output in E₂ + MPA-treated first trimester decidual cell monolayers. Confluent decidual cells were incubated for 7 days in E₂ + MPA and then switched to DM with corresponding steroids alone or with 2.5 U/ml thrombin, or 10 ng/ml TNF α or IL-1 β for 24 hours. Levels of sFlt-1 and VEGF were measured by ELISA in conditioned DM and normalized to cell protein (details in Materials and Methods). $n = 9$, mean $\pm$ SEM. *Versus sFlt-1 fold change for TNF α or IL-1 β , $P < 0.05$. **Versus VEGF fold change for thrombin, TNF α , or IL-1 β ; $P < 0.05$.

reversed by the addition of either VEGF or PIGF.³² Depressed plasma VEGF and PIGF levels and elevated sFlt-1 levels in the circulation^{34,35} but not in the amniotic fluid³⁵ precede the appearance of preeclamptic symptoms. Levels of sFlt-1 are also elevated in preeclamptic placentae.^{16,32,36} The urine of severely preeclamptic patients contains elevated sFlt-1 and depressed angiogenic factor levels.³⁷ Consistent with a high incidence of preeclampsia in first pregnancies, serum levels of sFlt-1 are significantly higher in first compared with second pregnancies.³⁸

Resolution of the clinical features of preeclampsia is accompanied by a precipitous fall in circulating sFlt-1 levels after delivery of the placenta,³⁹ suggesting the placenta is the primary source of circulating sFlt-1 during preeclampsia. However, levels of circulating sFlt-1 remain elevated in the circulation of women with preeclampsia for more than a year after delivery, indicating an extraplacental source of excess sFlt-1 production.⁴⁰ The current findings, taken together with a report that plasma sFlt-1 levels are higher in the uterine than antecubital vein in women with preeclampsia,⁴¹ point to the decidua as an additional source of excess sFlt-1 found in the uteroplacental circulation of pregnant preeclamptic patients and the persistence of sFlt-1 in their systemic circulations postpartum. Although term decidual cells do not respond to thrombin with enhanced sFlt-1 production, given the amount of basal sFlt-1 production in cultured decidua and the amount of decidua present in a term uterus, it is likely that the decidua is a contributing source of daily circulating sFlt-1 in pregnancy and postpartum period. Recently, Nagamatsu et al⁴² found that hypoxia-induced VEGF output in cytotrophoblasts was limited to VEGF bound to sFlt-1, ie, inactive VEGF. These results suggest that placental hypoxia interferes with trophoblast-mediated vascular transformation by preferentially enhancing output of VEGF-neutralizing sFlt-1.³² Nevertheless, Karumanchi and Bdolah³⁹ noted that these *in vitro* studies did not identify the origin of the hypoxia that preferentially enhances sFlt-1 expression in the cytotrophoblasts suggesting that excess trophoblast sFlt-1 expression is probably a consequence, not a cause, of shallow placentation.

The current study focused on decidual cells, the predominant cell type at the implantation site,¹⁸ as a potential source of inhibition of endovascular trophoblast invasion via excess sFlt-1 expression. Leukocytes are highly abundant in first trimester decidua and are a rich source of cytokines and proteases.⁴³ To eliminate these potential confounding factors, isolated decidual cells were passaged until demonstrated by FACS to be free of the common leukocyte antigen CD45 as well as the monocyte marker CD14 before experimental use. These purified decidual cells were incubated with E₂ or E₂ plus MPA to mimic the hormonal milieu of pregnancy. The proinflammatory cytokines implicated in preeclampsia, TNF α or IL-1 β , or thrombin were then added to these cultures. Although MPA enhances plasminogen activator inhibitor-1²⁹ and inhibits stromelysin-1 expression⁴⁴ in first trimester decidual cells, the current study found that MPA did not affect sFlt-1 in these cultures. Secreted levels of sFlt-1

were also found to be unaltered by $\text{TNF}\alpha$ or $\text{IL-1}\beta$ in the current study, despite previous observations that both cytokines markedly up-regulated interleukin-8⁴⁵ and monocyte chemoattractant protein-1 mRNA and protein expression in the decidual cells.⁴⁶

In contrast with the lack of response to either $\text{TNF}\alpha$ or $\text{IL-1}\beta$, thrombin elevated sFlt-1 mRNA and protein levels by severalfold. This response was unaffected by MPA and blocked by hirudin, demonstrating the requirement for active thrombin. Consistent with these *in vitro* results, the current study found that microdissected decidual cells express sFlt-1 mRNA, confirming that they can synthesize sFlt-1. Normal implantation is associated with thrombin-induced fibrin deposition in the absence of overt bleeding (Figure 1). Local generation of thrombin would be expected to be far greater in "preeclamptic" decidua, given the high incidence of underlying decidual hemorrhage as manifested by hemosiderin deposition as an indicator of occult bleeding,²³ intrauterine hematoma formation,²⁴ and the overt hemorrhage of abruption.^{25,26} Thus, Salafia and colleagues²³ evaluated 462 consecutive placentas of patients delivered at <32 weeks' gestation compared with 108 consecutive term placentas for evidence of prior decidual hemorrhage as manifested by hemosiderin deposition in the decidua basalis. Decidual hemosiderin was significantly more common in patients with preterm preeclampsia (45/76, 60%) compared with term controls (1/108, 0.8%). Nagy and associates²⁴ conducted a prospective cohort study of perinatal outcomes in 187 pregnant women with sonographic evidence of intrauterine hematomas in the first trimester compared with 6488 controls without hematomas and noted the former patients had an increased prevalence of preeclampsia (relative risk: 4.0; 95% confidence interval: 2.4–6.7). First trimester vaginal bleeding almost always stems from decidual hemorrhage is associated with both preeclampsia and fetal growth restriction.²⁵ Taken together, these findings provide a strong link between preeclampsia and first trimester decidual thrombin generation.

Maternal thrombophilias constitute an alternative source of excess thrombin. Severe but not mild preeclampsia has been consistently linked to inherited and acquired thrombophilias, which may reflect the far stronger association of severe preeclampsia with shallow placentation and fetal growth restriction.⁴⁷ Meta-analysis indicates that maternal heterozygosity for the factor V Leiden mutation carries a two- to threefold increased risk of severe preeclampsia.^{48,49} We and others demonstrated that maternal thrombophilias, such as the factor V Leiden mutation, are also associated with abruption.^{50,51} Thus, maternal thrombophilias provide a direct and indirect link to excess decidual thrombin generation to promote shallow placentation via excess sFlt-1 production.

Such excess sFlt-1 production at the implantation site has been proposed to impair pseudovasculogenesis by dysregulating angiogenesis.^{16,39} Thrombin-induced decidual sFlt-1 expression may interfere with VEGF-mediated local endometrial endothelial differentiation to block endovascular trophoblast invasion.³⁹ Indeed, Figures 7 and 8 show that thrombin exerts a preferential antiangio-

genic effect in first trimester decidua cells, but not term decidual cells, by up-regulating sFlt-1 but not VEGF. The absence of trophoblast invasion after 20 weeks' gestation^{3,4} suggests a lack of involvement of decidual cell-derived sFlt-1 in its action; thus expression at term would be expected to have no impact on trophoblast invasion.

In summary, the current results suggest that thrombin formed from underlying decidual hemorrhage and/or thrombophilias enhances sFlt-1 expression in first trimester decidual cells. Such sFlt-1 is proposed to impair pseudovasculogenesis by altering the local balance of angiogenic factors.^{16,39} The resulting restricted trophoblast invasion of the decidua would promote a pathological feed-forward cycle of impaired vascular transformation, leading to local hypoxia, which exacerbates the cycle by initiating the synthesis and release of sFlt-1 by cytotrophoblasts.⁴²

References

- Pijnenborg R, Bland JM, Robertson WB, Brosens I: Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy. *Placenta* 1983, 4:397–413
- Zhou Y, Damsky CH, Fisher SJ: Preeclampsia is associated with failure of human cytotrophoblasts to mimic a vascular adhesion phenotype: one cause of defective endovascular invasion in this syndrome. *J Clin Invest* 1997, 99:2152–2164
- Pijnenborg R: Trophoblast invasion. *Reprod Med Rev* 1994, 3:53–73
- Brosens IA, Robertson WB, Dixon HG: The role of the spiral arteries in the pathogenesis of preeclampsia. *Obstet Gynecol Annu* 1972, 1:177–191
- Lunell NO, Lewander R, Mamoun I, Nylund L, Sarby S, Thornstrom S: Uteroplacental blood flow in pregnancy induced hypertension. *Scand J Clin Lab Invest Suppl* 1984, 169:28–35
- Caniggia I, Mostachfi H, Winter J, Gassmann M, Lye SJ, Kuliszewski M, Post M: Hypoxia-inducible factor-1 mediates the biological effects of oxygen on human trophoblast differentiation through TGF β (3). *J Clin Invest* 2000, 105:577–587
- Rajakumar A, Whitelock KA, Weissfeld LA, Daftary AR, Markovic N, Conrad KP: Selective overexpression of the hypoxia-inducible transcription factor, HIF-2 α , in placentas from women with preeclampsia. *Biol Reprod* 2001, 64:499–506
- Lash GE, Taylor CM, Trew AJ, Cooper S, Anthony FW, Wheeler T, Baker PN: Vascular endothelial growth factor and placental growth factor release in cultured trophoblast cells under different oxygen tensions. *Growth Factors* 2002, 20:189–196
- Soleymanlou N, Jurisica I, Nevo O, Ietta F, Zhang X, Zamudio S, Post M, Caniggia I: Molecular evidence of placental hypoxia in preeclampsia. *J Clin Endocrinol Metab* 2005, 90:4299–4308
- Taylor RN, Grimwood J, Taylor RS, McMaster MT, Fisher SJ, North RA: Longitudinal serum concentrations of placental growth factor: evidence for abnormal placental angiogenesis in pathologic pregnancies. *Am J Obstet Gynecol* 2003, 188:177–182
- Thadhani R, Mutter WP, Wolf M, Levine RJ, Taylor RN, Sukhatme VP, Ecker J, Karumanchi SA: First trimester placental growth factor and soluble fms-like tyrosine kinase 1 and risk for preeclampsia. *J Clin Endocrinol Metab* 2004, 89:770–775
- Nadar SK, Karalis I, Al Yemeni E, Blann AD, Lip GY: Plasma markers of angiogenesis in pregnancy induced hypertension. *Thromb Haemost* 2005, 94:1071–1076
- Chaiworapongsa T, Romero R, Kim YM, Kim GJ, Kim MR, Espinoza J, Bujold E, Goncalves L, Gomez R, Edwin S, Mazor M: Plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated prior to the clinical diagnosis of pre-eclampsia. *J Matern Fetal Neonatal Med* 2005, 17:3–18
- Burri PH, Hlushchuk R, Djonov V: Intussusceptive angiogenesis: its emergence, its characteristics, and its significance. *Dev Dyn* 2004, 231:474–488
- Clark DE, Smith SK, He Y, Day KA, Licence DR, Corps AN, Lamm-

- glia R, Charnock-Jones DS: A vascular endothelial growth factor antagonist is produced by the human placenta and released into the maternal circulation. *Biol Reprod* 1998, 59:1540–1548
16. Zhou Y, McMaster M, Woo K, Janatpour M, Perry J, Karpanen T, Alitalo K, Damsky C, Fisher SJ: Vascular endothelial growth factor ligands and receptors that regulate human cytotrophoblast survival are dysregulated in severe preeclampsia and hemolysis, elevated liver enzymes, and low platelets syndrome. *Am J Pathol* 2002, 160:1405–1423
17. Ng YS, Ramsauer M, Loureiro RM, D'Amore PA: Identification of genes involved in VEGF-mediated vascular morphogenesis using embryonic stem cell-derived cystic embryoid bodies. *Lab Invest* 2004, 84:1209–1218
18. Dunn CL, Kelly RW, Critchley HO: Decidualization of the human endometrial stromal cell: an enigmatic transformation. *Reprod Biomed Online* 2003, 7:151–161
19. Rinehart BK, Terrone DA, Lagoo-Deenadayan S, Barber WH, Hale EA, Martin Jr JN, Bennett WA: Expression of the placental cytokines tumor necrosis factor alpha, interleukin 1beta, and interleukin 10 is increased in preeclampsia. *Am J Obstet Gynecol* 1999, 181:915–920
20. Hefler LA, Tempfer CB, Gregg AR: Polymorphisms within the interleukin-1 beta gene cluster and preeclampsia. *Obstet Gynecol* 2001, 97:664–668
21. Reister F, Frank HG, Kingdom JC, Heyl W, Kaufmann P, Rath W, Huppertz B: Macrophage-induced apoptosis limits endovascular trophoblast invasion in the uterine wall of preeclamptic women. *Lab Invest* 2001, 81:1143–1152
22. Bauer S, Pollheimer J, Hartmann J, Husslein P, Aplin JD, Knofler M: Tumor necrosis factor-alpha inhibits trophoblast migration through elevation of plasminogen activator inhibitor-1 in first-trimester villous explant cultures. *J Clin Endocrinol Metab* 2004, 89:812–822
23. Salafia CM, Lopez-Zeno JA, Sherer DM, Whittington SS, Minior VK, Vintzileos AM: Histologic evidence of old intrauterine bleeding is more frequent in prematurity. *Am J Obstet Gynecol* 1995, 173:1065–1070
24. Nagy S, Bush M, Stone J, Lapinski RH, Gardo S: Clinical significance of subchorionic and retroplacental hematomas detected in the first trimester of pregnancy. *Obstet Gynecol* 2003, 102:94–100
25. Weiss JL, Malone FD, Vidaver J, Ball RH, Nyberg DA, Comstock CH, Hankins GD, Berkowitz RL, Gross SJ, Dugoff L, Timor-Tritsch IE, D'Alton ME, FASTER Consortium: Threatened abortion: a risk factor for poor pregnancy outcome, a population-based screening study. *Am J Obstet Gynecol* 2004, 190:745–750
26. Aoyama K, Suzuki Y, Sato T, Yamamoto T, Kojima K, Usami T, Ohte N, Suzumori K: Cardiac failure caused by severe pre-eclampsia with placental abruption, and its treatment with anti-hypertensive drugs. *J Obstet Gynaecol Res* 2003, 29:339–342
27. Rosen T, Kuczyński E, O'Neill LM, Funai EF, Lockwood CJ: Plasma levels of thrombin-antithrombin complexes predict preterm premature rupture of the fetal membranes. *J Matern Fetal Med* 2001, 10:297–300
28. Lockwood CJ, Nemerson Y, Guller S, Krikun G, Alvarez M, Hausknecht V, Gurpide E, Schatz F: Progestational regulation of human endometrial stromal cell tissue factor expression during decidualization. *J Clin Endocrinol Metab* 1993, 76:231–236
29. Schatz F, Lockwood CJ: Progesterone regulation of plasminogen activator inhibitor type 1 in primary cultures of endometrial stromal and decidual cells. *J Clin Endocrinol Metab* 1993, 77:621–625
30. Arici A, Marshburn PB, MacDonald PC, Dombrowski RA: Progesterone metabolism in human endometrial stromal and gland cells in culture. *Steroids* 1999, 64:530–534
31. Roberts JM, Taylor RN, Musci TJ, Rodgers GM, Hubel CA, McLaughlin MK: Preeclampsia: an endothelial cell disorder. *Am J Obstet Gynecol* 1989, 161:1200–1204
32. Maynard SE, Min JY, Merchan J, Lim KH, Li J, Mondal S, Libermann TA, Morgan JP, Selke FW, Stillman IE, Epstein FH, Sukhatme VP, Karumanchi SA: Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *J Clin Invest* 2003, 111:649–658
33. Eremina V, Sood M, Haigh J, Nagy A, Lajoie G, Ferrara N, Gerber HP, Kikkawa Y, Miner JH, Quaggin SE: Glomerular-specific alterations of VEGF-A expression lead to distinct congenital and acquired renal diseases. *J Clin Invest* 2003, 111:707–716
34. Levine RJ, Maynard SE, Qian C, Lim KH, England LJ, Yu KF, Schisterman EF, Thadhani R, Sachs BP, Epstein FH, Sibai BM, Sukhatme VP, Karumanchi SA: Circulating angiogenic factors and the risk of preeclampsia. *N Engl J Med* 2004, 350:672–683
35. Park CW, Park JS, Shim SS, Jun JK, Yoon BH, Romero R: An elevated maternal plasma, but not amniotic fluid, soluble fms-like tyrosine kinase-1 (sFlt-1) at the time of mid-trimester genetic amniocentesis is a risk factor for preeclampsia. *Am J Obstet Gynecol* 2005, 193:984–989
36. Tsatsaris V, Goffin F, Munaut C, Brichant JF, Pignon MR, Noel A, Schaaps JP, Cabrol D, Franckenne F, Foidart JM: Overexpression of the soluble vascular endothelial growth factor receptor in preeclamptic patients: pathophysiological consequences. *J Clin Endocrinol Metab* 2003, 88:5555–5563
37. Buhimschi CS, Norwitz ER, Funai E, Richman S, Guller S, Lockwood CJ, Buhimschi IA: Urinary angiogenic factors cluster hypertensive disorders and identify women with severe preeclampsia. *Am J Obstet Gynecol* 2005, 192:734–741
38. Wolf M, Shah A, Lam C, Martinez A, Smirnakis KV, Epstein FH, Taylor RN, Ecker JL, Karumanchi SA, Thadhani R: Circulating levels of the antiangiogenic marker sFLT-1 are increased in first versus second pregnancies. *Am J Obstet Gynecol* 2005, 193:16–22
39. Karumanchi SA, Bdelah Y: Hypoxia and sFlt-1 in preeclampsia: the "chicken-and-egg" question. *Endocrinology* 2004, 145:4835–4837
40. Wolf M, Hubel CA, Lam C, Sampson M, Ecker JL, Ness RB, Rajakumar A, Daftary A, Shakir AS, Seely EW, Roberts JM, Sukhatme VP, Karumanchi SA, Thadhani R: Preeclampsia and future cardiovascular disease: potential role of altered angiogenesis and insulin resistance. *J Clin Endocrinol Metab* 2004, 89:6239–6243
41. Bujold E, Romero R, Chaiworapongsa T, Kim YM, Kim MR, Espinoza J, Goncalves LF, Edwin S, Mazor M: Evidence supporting that the excess of the sVEGFR-1 concentration in maternal plasma in preeclampsia has a uterine origin. *J Matern Fetal Neonatal Med* 2005, 18:9–16
42. Nagamatsu T, Fujii T, Kusumi M, Zou L, Yamashita T, Osuga Y, Momoeda M, Kozuma S, Taketani Y: Cytotrophoblasts up-regulate soluble fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia. *Endocrinology* 2004, 145:4838–4845
43. Salamonsen LA, Lathbury LJ: Endometrial leukocytes and menstruation. *Hum Reprod Update* 2000, 6:16–27
44. Schatz F, Papp C, Toth-Pal E, Lockwood CJ: Ovarian steroid-modulated stromelysin-1 expression in human endometrial stromal and decidual cells. *J Clin Endocrinol Metab* 1994, 78:1467–1472
45. Lockwood CJ, Paidas M, Krikun G, Koopman LA, Masch R, Kuczyński E, Kliman H, Baergen RN, Schatz F: Inflammatory cytokine and thrombin regulation of interleukin-8 and intercellular adhesion molecule-1 expression in first trimester human decidua. *J Clin Endocrinol Metab* 2005, 90:4710–4715
46. Lockwood CJ, Matta P, Krikun G, Koopman LA, Masch R, Toti P, Arcuri F, Huang S-T, Funai EF, Schatz F: Regulation of monocyte chemoattractant protein-1 expression by tumor necrosis factor alpha and interleukin-1 beta in first trimester human decidual cells: implications for preeclampsia. *Am J Pathol* 2006, 168:445–452
47. Vatten LJ, Skjaerven R: Is pre-eclampsia more than one disease. *Br J Obstet Gynecol* 2004, 111:298–302
48. Dudding TE, Attia J: The association between adverse pregnancy outcomes and maternal factor V Leiden genotype: a meta-analysis. *Thromb Haemost* 2004, 91:700–711
49. Lin J, August P: Genetic thrombophilias and preeclampsia: a meta-analysis. *Obstet Gynecol* 2005, 105:182–192
50. Roqué H, Paidas MJ, Funai EF, Kuczyński E, Lockwood CJ: Maternal thrombophilias are not associated with early pregnancy loss. *Thromb Haemost* 2004, 91:290–295
51. Alfirevic Z, Roberts D, Martlew V: How strong is the association between maternal thrombophilia and adverse pregnancy outcome? A systematic review. *Eur J Obstet Gynecol Reprod Biol* 2002, 101:6–14