A Novel Human-Specific Soluble Vascular Endothelial Growth Factor Receptor 1

Cell Type-Specific Splicing and Implications to Vascular Endothelial Growth Factor Homeostasis and Preeclampsia

Shay Sela, Ahuva Itin, Shira Natanson-Yaron, Caryn Greenfield, Debra Goldman-Wohl, Simcha Yagel, Eli Keshet

Abstract—A human-specific splicing variant of vascular endothelial growth factor (VEGF) receptor 1 (Flt1) was discovered, producing a soluble receptor (designated sFlt1-14) that is qualitatively different from the previously described soluble receptor (sFlt1) and functioning as a potent VEGF inhibitor. sFlt1-14 is generated in a cell type-specific fashion, primarily in nonendothelial cells. Notably, in vascular smooth muscle cells, all Flt1 messenger RNA is converted to sFlt1-14, whereas endothelial cells of the same human vessel express sFlt1. sFlt1-14 expression by vascular smooth muscle cells is dynamically regulated as evidenced by its upregulation on coculture with endothelial cells or direct exposure to VEGF. Increased production of soluble VEGF receptors during pregnancy is entirely attributable to induced expression of placental sFlt1-14 starting by the end of the first trimester. Expression is dramatically elevated in the placenta of women with preeclampsia, specifically induced in abnormal clusters of degenerative syncytiotrophoblasts known as syncytial knots, where it may undergo further messenger RNA editing. sFlt1-14 is the predominant VEGF-inhibiting protein produced by the preeclamptic placenta, accumulates in the circulation, and hence is capable of neutralizing VEGF in distant organs affected in preeclampsia. Together, these findings revealed a new natural VEGF inhibitor that has evolved in humans, possibly to protect nonendothelial cells from adverse VEGF signaling. Furthermore, the study uncovered the identity of a VEGF-blocking protein implicated in preeclampsia. (Circ Res. 2008;102:1566-1574.)

Key Words: VEGF ■ soluble VEGF receptor ■ splicing ■ preeclampsia ■ vascular smooth muscle cell

Vascular endothelial growth factor (VEGF) is the key factor promoting vasculogenesis and angiogenesis in the embryo and the factor orchestrating most, if not all, processes of adult neovascularization. In addition, VEGF performs nonvascular developmental functions and plays a number of homeostatic roles in the adult. The latter includes maintenance of endothelial fenestrations, a role in vasodilatation, and neurogenic and neurotrophic activities, among others. Not surprisingly, therefore, VEGF is under a tight spatial and temporal regulation, and even a moderate deviation from its exquisite dosage control or perturbation of its precise morphogenetic gradients is detrimental for proper development and organ homeostasis.54

Among the multiple layers of VEGF control, natural VEGF inhibitors, primarily soluble VEGF receptors, are likely to be important. Indeed, VEGF receptor-1 (Flt1) messenger RNA (mRNA) may undergo alternative splicing in a way that the encoded protein retains the ligand-binding domain but is devoid of the membrane-spanning and intracellular kinase domains, hence functioning as a VEGF-trapping soluble receptor. This secreted protein (designated sFlt1) is a potent inhibitor of both VEGF-A, VEGF-B, and placental growth factor.2 It is widely used as a research tool for VEGF inhibition and, more recently, also harnessed in clinical trials of VEGF blockade. However, surprisingly little is known concerning natural sFlt1 regulation. Likewise, the different arenas in which it may act in physiological titration of VEGF or in pathological neutralization of VEGF activity remain to be uncovered. A remarkable recent example for a physiological role of sFlt1 is the identification of sFlt1 as the factor responsible for preventing vascular invasion onto the cornea.3 The best documented case of a pathological role is the involvement of sFlt1 in preeclampsia (PE).67

PE, affecting 5% of pregnant women, is characterized by severe maternal hypertension, proteinuria, glomerular endotheiosis, and neurological symptoms. Although the underlying pathogenic mechanism of PE is largely unknown, sFlt1 was implicated in PE causation because of its abnormally high levels in the circulation of preeclamptic women.7 Furthermore, a seminal study by Maynard et al6 shows that systemic sFlt1 causes a PE-like syndrome in rats, presumably through negating constitutive VEGF function in the kidney.
Initially considered as an endothelial cell (EC)-specific receptor, Flt1 was subsequently detected also in a number of nonendothelial cells, thus extending the spectrum of cells directly responsive to VEGF. We reason that the splicing ratio of Flt1/sFlt1 should determine the extent to which a given cell will transmit or, conversely, resist VEGF signaling. We propose that dynamic changes in the splicing ratio may serve a regulatory function, in general, and that predominance of the soluble receptor might protect nonendothelial cells from adverse effects of VEGF, in particular. Experiments reported here are compatible with the proposition of a role for sFlt1 in the EC/vascular smooth muscle cell (VSMC) interface of the mature vessel wall. A search through human expressed sequence tag databases indicated that an additional truncated Flt1 RNA transcript may exist, as indeed verified in a very recent study by Thomas et al8 and also encountered in this study. Here, we show that this transcript is naturally translated and secreted and functions as a potent VEGF-blocking soluble receptor. This natural VEGF inhibitor, designated by us as sFlt1-14, evolved during primate evolution and is the predominant VEGF inhibitor produced by human nonendothelial cells. Importantly, we show that sFlt1-14 is also the VEGF-neutralizing protein produced by the placenta of women with PE.

Materials and Methods

cDNA Cloning

Rapid amplification of complementary DNA (cDNA) ends (RACE) was performed using a BD Smart RACE cDNA Amplification Kit (BD Biosciences; catalog no. 634914). For cloning/sequencing of sFlt1-14 3’ untranslated regions (UTRs), RNAs of PE placentas were reverse-transcribed using a primer complementary to the beginning of exon 14 of Flt1.

Recombinant sFlt1 and sFlt1-14 Proteins

cDNAs encompassing the entire coding region of both soluble receptor isoforms were subcloned into Bluescript expression vectors and transfected onto T7 polymerase-expressing Hela cells. Twenty to 24 hours later, media were collected and the cells were harvested. Secreted and cell-associated proteins were immunoprecipitated with the FLT11 antibody and analyzed by immunoblotting with the ab9540 antibody (Abcam), both antibodies directed against the extracellular domain of Flt1.

VEGF Inhibition Assay

Porcine aortic ECs engineered to express high levels of human VEGF-receptor 2 (VEGF-R2) (a gift from Prof Gera Neufeld, Technion, Haifa, Israel) were exposed to VEGF preincubated (or not) with sFlt1 or sFlt1-14 proteins. A reduction in VEGF-R2 phosphorylation was determined using antibodies detecting phospho-VEGF-R2 (Cell Signal-identification of a Novel Splice Variant of a Soluble VEGF Receptor-1 (sFlt1-14)

Vegetables and protein analyses were hybridized with 32P-labeled specific cDNA probes prepared with the aid of a Rediprime kit (Amersham). Probes derived from different regions of Flt1 transcripts were used, as specified in the text and figure legends. For in situ detection of sFlt1-14 RNA, paraffin-embedded sections of normal and PE placenta were hybridized with a 35S-labeled riboprobe composed of exon 15a sequences as previously described.

For immunoprecipitation/immunoblotting analysis, placenta were homogenized and immunoprecipitated with 1 of 3 antibodies: CHFK, CESS, and FLT11 (Sigma; catalog no. V4262) and incubated overnight with Protein A-coated beads (P3391, Sigma). Precipitates were then dissolved and proteins separated on 6% acrylamide gel, electrophoretically transferred to a membrane, and detected with the indicated antibodies. For analysis of serum proteins, 20 mL of serum from PE patients was first concentrated through capture on FLT11-coated beads, and affinity-purified proteins were analyzed by Western blotting as described above. Placental samples were taken with Institutional Review Board approval. PE samples met the American College of Obstetricians and Gynecologists criteria for severe PE.

Mass Spectrometry

Protein homogenate of a PE placenta was incubated for 3 hours with a rabbit preimmune serum (20 μL), incubated overnight with Protein A beads, and precipitated with 3 hours of incubation with 15 μL of the CESS antibody. Precipitated proteins were subsequently separated on a 6% acrylamide gel and visualized by staining with Coomassie blue. The 115- and 130-kDa bands were cut out, digested with trypsin, and subjected to mass spectrometric analysis by liquid chromatography/tandem mass spectrometry on DECA/LCQ. Peptides were identified and analyzed by Pep-Analyzer and Sequest software against the nr database of human, mouse, rat, bovine, and rabbit.

Enzyme-Linked Immunosorbent Assay (ELISA)

A commercial ELISA kit for the detection of all soluble VEGF receptor isoforms was used (R&D Systems; DVR 100B).

Cells and Culture

Human primary endothelial and VSMCs were a gift from Dr Flugelman Moshe (Technion). Primary cell isolation and culture details were previously described.

Immunohistochemistry

Paraffin-embedded sections of human corneas were used. Antigen retrieval was performed by microwave heating in pH 6 citrate buffer. The CESS antibody was used at a 1:100 dilution.

Antibodies

Rabbit polyclonal CESS and CHFK antibodies were generated by a rabbit preimmune serum (20 μL). Precipitated proteins were subsequently separated on a 6% acrylamide gel, electrophoretically transferred to a membrane, and detected with the indicated antibodies.

Statistics

Data were analyzed by 1-way analysis of variance, followed by Tukey post hoc tests.

Results

Identification of a Novel Splice Variant of a Soluble VEGF Receptor-1 (sFlt1-14)

The majority of mRNA molecules transcribed from VEGF-R1 DNA encodes a transmembrane signaling receptor, but a small fraction of primary transcripts is usually alternatively spliced, generating a truncated mRNA devoid of the transmembrane- and intracellular-encoding domains (Figure 1) and hence capable of sequestering VEGF and block signaling. In search of circumstances in which the soluble receptor (sFlt1) may fulfill a physiological role of VEGF inhibition, we first searched for dynamic changes in the Flt1/sFlt1 splicing ratio. To this end, RNA probes specific for the full receptor or for its soluble counterpart were used. Unexpectedly, there was marked discrepancy between results obtained using rodent or human materials. In certain human tissues, sFlt1 could not be detected with a sFlt1-specific probe (ie, probe detecting unique intron 13 sequences; see Figure 1; data not shown but see below). This led us to examine the possibility that human cells may express a
different form of soluble receptor. 3' RACE methodology was thus used for cloning truncated Flt1 mRNAs present in human placental RNA.

These studies culminated in the isolation and characterization of a novel human-specific splice variant of VEGF-R1, designated therein as sFlt1-14 (Figure 1A). A BLAT search of the University of California at Santa Cruz genome browser indeed revealed 3 expressed sequence tags likely to have been generated by alternative splicing different from that used to generate sFlt1. sFlt1-14 sequence data have been submitted to GenBank under accession no. EU368830. Nucleotide and amino acid alignment is presented in Figure I of the online data supplement, available at http://circres.ahajournals.org.

**sFlt1-14 Is a Natural VEGF Inhibitor**

Considering that sFlt1 and sFlt1-14 are qualitatively different proteins (with sFlt1-14 containing 75 amino acids not present in sFlt1 and with sFlt1 containing 31 highly conserved amino acids not present in sFlt1-14), it was essential to demonstrate that sFlt1-14 functions as a VEGF inhibitor. To this end, we first expressed a recombinant sFlt1-14 protein in human Hela cells (and for comparison, also produced sFlt1-expressing Hela cells). Amounts of the respective protein released to growth medium were then monitored by ELISA directed against a shared extracellular epitope. For both proteins, concentrations of 100 to 200 ng/mL were detected in the medium. Media were further analyzed by immunoprecipitation and Western blotting with 2 different shared (extracellular) antibodies, confirming the mutually exclusive production of either sFlt1-14 or sFlt1. Two sFlt1-14 proteins were detected: a cell-associated 115-kDa isoform and a secreted 130-kDa isoform, likely representing additional posttranslational modifications. As expected from the size of the respective coding region, sFlt1-14 proteins were larger than the corresponding secreted and cellular sFlt1 isoforms (Figure 2A). To determine whether sFlt1-14 inhibits VEGF signaling, increasing amounts were preincubated with a constant amount of VEGF (20 ng/mL) before adding to the growth medium of porcine aortic ECs engineered to express high levels of human VEGF-R2. Levels of VEGF-R2 phosphorylation were then measured as a function of the sFlt1-14/VEGF ratio. As shown in Figure 2B, sFlt1-14 inhibited VEGF-R2 phosphorylation almost completely already at a 1:1 ratio. We conclude that sFlt1-14 is a potent inhibitor of VEGF signaling and that its inhibitory activity is comparable with that of sFlt1.
sFlt1-14 Is Expressed by Corneal Epithelial Cells and Is Dynamically Regulated in VSMCs at the Human Vessel Wall

Although VEGF-R1 is primarily an endothelial-specific receptor, it is also expressed by certain nonendothelial cells like monocytes and dendritic cells. With our discovery of sFlt1-14, we wished to identify cell types in which it is expressed. The avascular cornea is currently the best example for a tissue protected against VEGF-primed vessel invasion by a soluble VEGF receptor.5 It was of interest, therefore, to determine whether sFlt1-14 is produced by human corneal cells. sFlt1-14 mRNA was indeed detected in epithelia isolated from human cornea by polymerase chain reaction (data not shown), and immunohistochemical analysis with a sFlt1-14–specific antibody detected sFlt1-14 protein in epithelial cells of human cornea (Figure 3A). The relative contribution of sFlt1 and sFlt1-14 for negating VEGF in the cornea remains to be elucidated.

The blood vessel wall is of particular interest considering that perturbations of VEGF-mediated vasodilatation are associated with impairment of blood pressure regulation. We reason, therefore, to determine whether sFlt1-14 is produced by human corneal cells, sFlt1-14 mRNA was indeed detected in epithelia isolated from human cornea by polymerase chain reaction (data not shown), and immunohistochemical analysis with a sFlt1-14–specific antibody detected sFlt1-14 protein in epithelial cells of human cornea (Figure 3A). The relative contribution of sFlt1 and sFlt1-14 for negating VEGF in the cornea remains to be elucidated.

The blood vessel wall is of particular interest considering that perturbations of VEGF-mediated vasodilatation are associated with impairment of blood pressure regulation. We reason, therefore, that natural VEGF inhibitors like sFlt1 or sFlt1-14 may play a role in maintaining VEGF homeostasis in the mature vessel wall. To test this proposition, we first separated a freshly excised human vessel, a saphenous vein, into its endothelial and VSMC components and examined expression of VEGF-R1 and its soluble isoforms in primary cultures thereof. Whereas ECs were found to express sFlt1 but not sFlt1-14, VSMCs from the same vessel expressed exclusively sFlt1-14 (Figure 3B). Identical dichotomy in soluble receptor expression was also detected in primary cultures derived from a human radial artery (data not shown). These results not only provide a first evidence for constitutive expression of a natural VEGF inhibitor in VSMCs but also show that sFlt1-14 is produced in a strictly cell-type specific manner. Furthermore, in VSMCs, almost all primary transcripts are converted into sFlt1-14, leaving only a minor fraction of mRNA from which the full-size receptor can be translated (Figure 3B). This is in sharp contrast to ECs, in which sFlt1 is vastly outnumbered by VEGF-R1 mRNA. Obviously, the ratio of transmembrane/soluble receptor is a critical parameter by shifting the balance from ability to transmit VEGF signals to negating them. Interestingly, dendritic cells also produce sFlt1-14 and not the previously described sFlt1, and again, this soluble receptor predominates over the transmembrane receptor (Figure 3C).

Next, we examined whether sFlt1-14 is dynamically regulated in VSMCs under conditions similar to those prevailing in the vessel wall, namely, proximity to ECs and exposure to EC-produced VEGF. sFlt1-14 expression was compared in VSMCs, EC monoculture, and in a VSMC/EC (1:1) coculture. As shown in Figure 3B, sFlt1-14 was strongly induced in the coculture, whereas expression of the endothelially produced Flt-1 and sFlt1 remained unchanged (probing in parallel the RNA blot with isoform-specific riboprobes confirmed the assigned identities [data not shown]). Addition of VEGF to VSMCs monocultures also led to upregulated sFlt1-14 expression but not of Flt1 or sFlt1 expression (Figure 3D and supplemental Figure III). These results are compatible with a putative, yet unknown, role of sFlt1-14 taking place in the EC/VSMC interface.

sFlt1-14 Is Upregulated in Syncytial Knots of the Preeclamptic Placenta

Previous studies highlighted the role of sFlt1 in PE causation, based on findings that sFlt1 accumulates the circulation of
women with PE to substantially higher levels than in normal pregnancies and on findings that when expressed in rats, a chimeric protein containing ligand-binding domain of sFlt1 produces the PE-like symptoms of hypertension and proteinuria. Yet, the placental cellular sources of circulating VEGF receptors and factors responsible for its deregulated expression in PE are not known. Furthermore, with the discovery of sFlt1-14, the question arises as to which soluble receptor is associated with PE. To address these questions, placental RNAs from different stages of normal pregnancies and from term placentas of both normal and PE pregnancies were first examined by Northern blot analysis with shared and specific riboprobes. Although expression of total soluble VEGF-R1 increases progressively, particularly toward the third trimester of pregnancy, the relative contribution of the 2 sFlt1 isoforms changed dramatically, with sFlt1-14 becoming the dominant isoform over time. Thus, whereas the “classic” sFlt1 accounted for approximately half of placenta-produced soluble receptors during the first trimester, a further increase at later times was entirely attributable to upregulated expression of sFlt1-14 (Figure 4A). Transition to almost exclusive production of sFlt1-14 took place by the beginning of the second trimester and was clearly evident at term (Figure 4A). sFlt1-14 mRNA was also the dominant, if not the sole, transcript encoding soluble Flt1 in the PE placenta, reaching steady-state levels exceedingly higher than in the term normal placenta (Figure 4B). Identity of the respective mRNAs was secured through probing with a sFlt1-14–specific riboprobe (supplemental Figure IV). sFlt1-14 was identified by direct sequencing of cDNA clones prepared from placenta RNA of 7 of 7 PE patients.

Whereas coding sFlt1-14 sequences were invariably identical in all cases, extensive RNA editing of the 3’-UTR was encountered (supplemental Figure V). To identify which cells in the PE placenta produce sFlt1-14, we carried out in situ hybridization analysis using a sFlt1-14–specific riboprobe (Figure 5). The major sites of placental
sFlt1-14 expression were found to be syncytial knots, i.e., clusters of degenerative syncytiotrophoblasts that are a histological hallmark of the PE placenta. Of note, sFlt1-14 is also produced by seemingly healthy syncytiotrophoblasts but at significantly lower levels and, importantly, not at all in vascular ECs (Figure 5, top middle, and at a higher magnification in the image on the top right), thus identifying syncytial knots as the predominant site of sFlt1-14 production. It should be pointed out that

Figure 4. Preferential expression of sFlt1-14 in the maturing normal and PE placenta. A, Left and Middle, Two representative experiments of Northern blot analysis of placental RNAs from the indicated weeks of gestation with the probe detecting shared sequences of sFlt1-14 and sFlt1. Right, The sFlt1-14/sFlt1 expression ratio calculated on the basis of hybridization intensities. Note dominance of sFlt1-14 from the second trimester and onward. Cases analyzed were from the following gestational weeks: first trimester: 8, 9, 9, 10, 11 (n=5); second trimester: 13, 15, 15 (n=3); term placenta: 38, 38, 39, 39, 40 (n=6). *P<0.02 compared with the first trimester. Data are presented as mean±SEM. B, Northern blot analysis with the shared extracellular part of Flt1 identifies sFlt1-14 as the predominant receptor isoform in the PE placenta.

Figure 5. Identification of sFlt1-14–producing cell in the placenta. Top, In situ hybridization with a sFlt1-14–specific riboprobe. Note that syncytial knots (SK) are the major source of placental sFlt1-14, with a lower expression by apparently healthy syncytiotrophoblasts (STB) and no sFlt1-14 expression by placental ECs. Hybridization signal is evident as black autoradiographic grains. Bottom, Serial sections hybridized with either sFlt1-14–specific probe (left) or a sFlt1–specific probe (right). Note extensive syncytial knots hybridization using sFlt1-14 but near background signal with sFlt1. The latter probe gave a clear hybridization signal in syncytiotrophoblasts of first trimester placenta, indicative of its potency (data not shown).
our RNA data (Figure 4A) but differs from the findings of (supplemental Figure VI). Of note, this finding is consistent with domination/immunoblotting experiments using different constellations placenta (Figure 6A). Furthermore, through immunoprecipita-
tion data, sFlt1-14 protein was abundantly produced in term
pregnancies were examined for the relative abundance of soluble
receptors, using immunoprecipitations and immunoblotting with
3 indicated antibodies detecting each a different epitope of sFlt1-14 and immunoblotted with the antibody unique to sFlt1-
14. This procedure identified the 130- and 115-kDa placent
al proteins as sFlt1-14. B, Serum (20 mL) of a PE patient were
passed through a column of beads coated with anti-Flt1 antibi-
obodies (against a shared extracellular epitope), eluted at a low
pH, and immunoblotted with the sFlt1-14-specific CESS anti-
body. For comparison, a CESS immunoprecipitate of the pla-
centa was run in parallel and immunoblotted. Note that the
same 2 protein bands detected in the placenta (and identified
above as sFlt1-14 proteins) are also found in the PE serum.

Discussion

The study uncovered a new player in VEGF regulation, namely,
a novel variant of soluble VEGF receptor-1 (designated sFlt1-
14) functioning as a potent VEGF inhibitor. Evolutionarily,
sFlt1-14 represents a relatively recent addition to the multiple
layers of VEGF control, because the unique splicing pattern
generating it, using a previously unrecognized intron 14 splice acceptor site, only takes place in humans and possibly also in nonhuman primates. Its fixation in the human genome should have a profound affect on VEGF homeostasis in adult human organs. This is attributable to the fact that the 3 possible fates of primary VEGF-R1 transcripts, namely, conversion to mRNAs encoding either the full-size signaling receptor or 1 of the 2 truncated soluble receptors, are mutually exclusive. Thus, the highly preferred utilization of the intron 14 splice acceptor site in nonendothelial cells reported here not only produces a strong VEGF inhibitor but also comes on the expanse of the possibility to transmit VEGF-
R1–mediated signals altogether. This is in contrast to sFlt1, whose production in ECs is in most cases accompanied by a large excess of the transmembrane receptor.

Several additional findings support the notion that sFlt1-14 may protect nonendothelial cells from unwarranted VEGF sig-
naling. In the cornea, a protective role for soluble Flt1 receptor against VEGF-induced neovascularization has been convinc-
ingly shown, and we show here that sFlt1-14 is likely to participate in this protection. In the context of the blood vessel wall, several considerations support the notion of sFlt1-14–mediated protection. First, there is a clear separation of sFlt1 and sFlt1-14 expression in ECs and nonendothelial cells, respec-
tively. In the vessel wall, this dichotomy is evidenced by sFlt1 expression in ECs and sFlt1-14 exclusively in neighboring

Figure 6. sFlt1-14 protein produced by the placenta accumu-
lates in the circulation of PE patients. A, Protein extracts from
term human placenta were immunoprecipitated separately with the
3 indicated antibodies detecting each a different epitope of sFlt1-14 and immunoblotted with the antibody unique to sFlt1-
14. This procedure identified the 130- and 115-kDa placent
al proteins as sFlt1-14. B, Serum (20 mL) of a PE patient were
passed through a column of beads coated with anti-Flt1 antibi-
obodies (against a shared extracellular epitope), eluted at a low
pH, and immunoblotted with the sFlt1-14-specific CESS anti-
body. For comparison, a CESS immunoprecipitate of the pla-
centa was run in parallel and immunoblotted. Note that the
same 2 protein bands detected in the placenta (and identified
above as sFlt1-14 proteins) are also found in the PE serum.

sFlt1-14 Accumulates in the Circulation of
PE Patients

Findings that the PE placenta expresses primarily sFlt1-14 argue that this is the major soluble receptor isoform capable of accumulating in the serum of PE patients. To show that sFlt1-14 protein is produced in the PE placenta and subsequently released into the circulation of PE patients, the following experiments were performed. First, term placentas from normal and PE pregnancies were examined for the relative abundance of soluble receptors, using immunoprecipitations and immunoblotting with isoform-specific antibodies. As expected from the mRNA expression data, sFlt1-14 protein was abundantly produced in term placenta (Figure 6A). Furthermore, through immunoprecipita-
tion/immunoblotting experiments using different constellations
of common and unique antibodies, we could show the domi-
nance of sFlt1-14 protein over sFlt1 protein in term placenta
(supplemental Figure VI). Of note, this finding is consistent with our RNA data (Figure 4A) but differs from the findings of Thomas et al who detected comparable levels of sFlt1-14 and sFlt1 in placenta. Second, proteins from a preeclamptic placenta were immunoprecipitated with the CESS sFlt1-14--specific anti-
tbody, and the eluted 115-kDa band was unequivocally identi-
fied as sFlt1-14 using mass spectrometry. Of a particular
diagnostic value was a peptide spanning the exon 13/exon 14
junction because it is present in sFlt1-14 but missing in sFlt1 and because the smaller size of this band rules out that it represents the full-size Flt1 receptor (supplemental Figure VII). It should be pointed out that because all peptides identified by mass spectrometry are shared by full-size Flt1, it could, in principle, represent a proteolytic cleavage product of Flt1. This is unlikely, however, considering that the analyzed protein band was immu-
no precipitated with the sFlt1-14--specific antibody CESS (rec-
ognizing an epitope missing in Flt1) and further confirmed to include the CESS epitope by an analytic CESS immunoblotting
done in parallel before its elution from the gel. Initial analysis of the 130-kDa band identified this band also as a truncated Flt1 receptor. Third, sera of PE women were analyzed for soluble Flt1 receptors. ELISA with antibody directed against a shared extracellular domain confirmed an earlier report of significantly elevated levels of circulating soluble receptor in PE (800 to 900 pg/mL versus 8000 to 10 000 pg/mL in control and PE third trimester sera, respectively). To identify the molecular identity of circulating soluble receptors, PE serum specimens were passed through Flt1 antibody-coated columns and bound proteins were subsequently analyzed by western blotting (Figure 6B). Again, sFlt1-14 protein was readily detected in the PE serum, visualized as 2 bands identical in size to those produced by the cells transfected with sFlt1-14 expression plasmid and detected with the sFlt1-14--specific antibody (Figure 2A). Im-
munoblotting the PE serum with an antibody directed against an extracellular epitope that recognizes all soluble Flt1 isoforms failed to present other than the 2 sFlt1-14 bands (supplemental Figure VIII).

 syncytial knots are often found in aged normal placenta, and we could indeed detect sFlt1-14 expression in syncytial knots of normal placenta (data not shown). We conclude that the much increased abundance of syncytial knot in PE accounts for the greatly elevated levels of sFlt1-14 produced in the PE placenta.
VSMCs. Second, sFlt1-14 is dynamically regulated by proximity to ECs and by VEGF directly, compatible with the notion that sFlt1-14 may physiologically function in titration of surplus VEGF. The role(s) of VEGF constitutively produced by the mature, healthy endothelium is poorly understood. For example, it is not clear whether perturbing normal function of endothelially produced VEGF may account for the hypertension developing in cancer patients treated with anti-VEGF antibodies. We propose that soluble VEGF-R1 elaborated by VSMCs may keep in check the levels of VEGF acting in a paracrine fashion (like preventing VEGF-induced VSMC migration) while leaving autocrine VEGF functions unaffected. It should be pointed out that in mice, VSMCs abundantly express Flt1 rather than sFlt1-14 (supplemental Figure IX), arguing that a putative protective role for a VEGF-sequestering soluble receptor in the vessel wall is not restricted to humans.

The full repertoire of nonendothelial cells expressing sFlt1 remains to be elucidated. Of note, sFlt1-14 is also expressed by certain human myeloid cells alongside VEGF-R1. The significance of VEGF-R1-mediated VEGF signaling in these cells and, accordingly, possible modulations by the soluble receptor also need to be determined. The newly discovered sFlt1-14 is qualitatively different from sFlt1 by both missing 31 amino acids and containing exon 14 coded amino acids, as well as 28 unique amino acids. Although the 31 amino acids missing in sFlt1-14 are evolutionary highly conserved, arguing for their functional importance, inhibition of VEGF signaling was not compromised. Conversely, it is possible that addition of extra amino acids (e.g., a polyserine stretch) confer unique biological properties to sFlt1-14. Likewise, the completely different 3’-UTR may strongly impact on a differential sFlt1-14 regulation. The UTR is known to affect many aspects of mRNA function, including nuclear export, cytoplasmic localization, and translational efficiency and stability and even to determine tissue-specific functions. These parameters may be further modified by posttranscriptional RNA editing. Alu insertions, in particular, are known to provide a favored substrate for RNA editing, as also demonstrated here for the Alu element nested in the 3’-UTR of sFlt1-14. In the case reported here, extensive RNA editing was accomplished by exceedingly high steady-state levels of sFlt1-14 mRNA in the placenta and was also associated with clinical manifestations of intrauterine growth restriction. Because we failed to detect RNA editing in additional PE cases not complicated by intrauterine growth restriction, it is tempting to speculate that these phenotypes are causally related, but this must of course be substantiated by analysis of more cases. The most significant pathology in which a soluble VEGF-R1 has been causally implicated is PE. This was based on detecting the specific disease and that sFlt1-14 is a human-specific protein raises the speculation that a unique aspect in sFlt1-14 regulation or a unique, yet unidentified, biological property of this protein is responsible for disease. Yet, the reason why rodents do not develop PE could also be attributable to inherent differences in placenta in rodents and primates. A sFlt1-14–expressing mouse (currently under construction) should aid in addressing this question. Here, we identified syncytial knots as the major source of local and circulating sFlt1-14. The fact that these structures increase in number in the normal aging placenta but are much more abundant in the degenerating PE placenta, a phenomenon known as the “Tenney–Parker change,” may explain findings that serum levels of soluble receptors (now identified as mostly sFlt1-14) increase in the second and third trimesters of normal pregnancies but to dramatically higher levels in PE pregnancies. These findings place syncytial knotting, a phenomenon common to several placental pathologies, as central to PE pathogenesis. We note in this regard that hypoxia, often considered to be an etiological cause of PE, is known to induce syncytial knotting and that syncytial knotting is also increased in placental malaria, known to be associated with increasing levels of circulating VEGF-R1.

Finally, we note that our findings provide another example for a required caution in extrapolating from mouse systems to human disease, because the latter might also involve human-specific mRNAs and proteins.

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Disclosures

None.

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Legend to Supplementary Figures

Online Figure I: sFlt1-14 coding and 3' UTR sequences. sFlt1-14 cDNA containing the full coding region and the 3'-UTR was cloned from human term placenta. Sequence was deposited at Genbank databases (accession No. EU368830).

Online Figure II: Characterization of CESS & CHFK antibodies. Antibodies specificity was confirmed by western blotting of recombinant proteins produced by HeLa cells transfected with either sFlt1- or sFlt1-14 encoding plasmids. Note that, whereas the common ab9540 antibody recognized both soluble receptors, our CESS and CHFK antibodies recognized only sFlt1-14.

Online Figure III: sFlt1-14 up regulation in VSMCs in response to VEGF. VEGF was added to cultures of VSMCs from a human saphenous vein for the indicated times as described in figure 3D, and sFlt1-14 mRNA detected by real time PCR using specific primers. Note up regulation by VEGF of constitutively expressed sFlt1-14. Specific primers were used for Flt1 and sFlt1 as well, but these transcripts were barely detectable. n=4; *=p<0.05, **=p<0.0001 compared to all other groups; Data presented as mean ± SEM. PCR primer sequences were the following: sFlt1-14 F- CTCCTGCGAAACCTCAGTG; sFlt1-14 R- GACGATGGTGACGTTGATGT; Flt1 F- TGGACTGACAGCAAACCCAA; Flt1 R- CAGAAACTGCGCCTGCTGAC.

Online Figure IV: Identification of the 3.0 Kb Flt transcript as sFlt1-14. RNA blots were analyzed with a probe detecting either exons 1-5 (shared by Flt1, sFlt1 and sFlt1-14) or exon 15a (unique to sFlt1-14). A. RNA from 9 weeks placenta (same as in Exp. 1 in figure 4A). B. RNA from two preparations of explanted placental villi of the 1st trimester. C. RNAs from twin placentas of a PE-complicated pregnancy (one of the twin embryos also diagnosed as IUGR (same RNAs used in figure 4C).

Online Figure V: sFlt1-14 3' UTR is edited in placenta of a PE patient complicated by IUGR. A woman with severe PE carried twins with the placenta of one of them accounting for most of the extremely high levels of circulating soluble receptor detected in her serum (8200pg/ml). (Left) sFlt1-14 was identified as the dominant soluble receptor expressed in the placenta of both twins, with the placenta of the twin also showing Intra-Uterine Growth Retardation (IUGR) expressing exceedingly high levels of sFlt1-14. (Right) The 3'UTR regions of sFlt1-14 mRNA from both placenta were cloned and cDNA sequences were determined. Shown is the 3'-UTR sequence (starting at the translation stop codon highlighted in bold) of the twin with IUGR. The Alu repeat is underlined and the 18 A-to-I transitions resulting from RNA editing events are marked with the letter "g". No editing events were detected in the 3'-UTR of the unaffected twin. To what extent, these structural alterations may affect biological regulation of sFlt1-14 remains to be determined.

Online Figure VI: Analysis of soluble Flt1 receptors in term placenta. Placental extracts from the indicated weeks of gestation were immuno-precipitated with the FLT11 antibody, and analyzed on western blots first with CESS and sequentially with ab9540. Note that the 130Kd and 115Kd sFlt1-14 proteins revealed by CESS (arrowheads) are also the prominent two bands detected by ab9540 which should
detect also sFlt1, thereby indicating that sFlt1-14 is the major soluble receptor produced by term placenta.

**Online Figure VII: sFlt1-14 identification by Mass spectrometry.** The 115 Kd protein band was immuno-precipitated from a preeclamptic placental biopsy with the CESS antibody and visualized by coomassie blue staining, eluted and analyzed by mass spectrometry. A part of the amino acids sequence of the membrane spanning Flt1 is presented for illustration. Highlighted in red are three identified peptides that map either to the first 13 exons or to the exon13/exon14 junction (Junction indicated by the green vertical line). The latter rule-out that the protein is sFlt1, while the CESS immuno-precipitation and size considerations rule-out that it is Flt1, thus identifying the 115kd protein unequivocally as sFlt1-14.

**Online Figure VIII: Analysis of soluble Flt1 receptors in PE serum.** The PE serum analyzed in Figure 6B using the CESS antibody for immuno-blotting was immuno-blotted here with ab9540 (targeting an extra cellular epitope) and bands were examined against those immunorecipitated by CESS from a term placenta. Note that the two bands detected in the PE serum (arrowheads) are similar in size to the two sFlt1-14 bands shown in Figure 6B. Importantly, no major additional bands were detected with ab9540, suggesting that sFlt1-14 is a major circulating receptor in PE.

**Online Figure IX: sFlt1 expression in mouse Vascular Smooth Muscle Cells (VSMCs).** A section of murine heart immuno-stained with sFlt1 specific antibody. Note presence of sFlt1 in VSMCs of coronary vessels but not in endothelial cells of the same vessels.
IB: CHFK  CESS  ab9540

130Kd
95Kd
Online Figure III
A. Probe Targeting region: Exons 1-5 Exon 15a

B. Probe Targeting region: Exons 1-5 Exon 15a

C. Probe Targeting region: Exons 1-5 Exon 15a

Online Figure IV
Online Figure VI

IB: CESS, ab9540

IP: FLT11 in all

Week 39
- 130Kd
- 95Kd
- 72Kd

Week 40
- 130Kd
- 95Kd
- 72Kd
Online Figure VIII