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A Vascular Endothelial Growth Factor Antagonist Is Produced by the Human Placenta and Released into the Maternal Circulation[1]

Dawn E. Clark,[3] Stephen K. Smith,[3] Yulong He,[3] Kate A. Day,[3] Diana R. Licence,[3] Anthony N. Corps,[3] Rosemarie Lammoglia,[4] and D. Stephen Charnock-Jones[2,3]

Reproductive Molecular Research Group,[3] Department of Obstetrics and Gynaecology, University of Cambridge, The Rosie Hospital, CB2 2SW, United Kingdom
Metris Therapeutics Ltd.,[4] Strangeways Research Laboratory, Worts Causeway, Cambridge, CB1 8RN, United Kingdom

ABSTRACT

Vascular endothelial growth factor (VEGF) is a potent secreted factor that promotes angiogenesis and maintains the integrity of the endothelium. Levels of VEGF are increased in many tumors and are elevated in women with pre-eclampsia, a serious disease of pregnancy. Here we show by in situ hybridization that the trophoblast contains the mRNA encoding a soluble version of the VEGF receptor known as Flt-1 (sFlt-1: initially described by Kendall and Thomas, PNAS 90:10705–10709). Binding assays and Western blotting of villus-conditioned media confirmed the production of sFlt-1. Serum from pregnant women was found to contain a VEGF-binding protein that was not present in serum from men or nonpregnant women. As determined by heparin affinity, column fractionation, and cross-linking, this protein was identical to sFlt-1. Taken together, these results show that the placenta secretes sFlt-1, which would be expected to be a VEGF antagonist. This is the first report of production of the sFlt-1 receptor in vivo, and it reveals a new mechanism for naturally regulating this potent angiogenic agent. The presence of such an antagonist suggests that regulation of VEGF action is essential to successful pregnancy. This has important implications for the activity of VEGF locally and systemically in other conditions.

INTRODUCTION

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a disulfide-linked homodimeric protein of between 24 and 46 kDa with five differentially spliced forms [1, 2]. It is a potent stimulator of angiogenesis in both the chick chorioallantoic membrane and rabbit cornea assays [3] and promotes endothelial cell proliferation and migration. VEGF is widely expressed in adult tissues and may play a role in the maintenance of the endothelium [4]. It is overexpressed in a variety of pathological conditions, particularly solid tumors, whose growth can be prevented by the inhibition of VEGF action [5, 6]. Transgenic mice bearing heterozygous and homozygous deletions of the VEGF gene fail to survive beyond embryonic Day 12, showing that VEGF is essential for embryonic development [7, 8] and that other closely related growth factors [9–11] cannot compensate for even its partial loss.

The receptors for VEGF—the *fms*-like tyrosine kinase, Flt-1 [12], and the kinase domain receptor, KDR [13]—are restricted in their expression patterns. While both receptors are present on endothelial cells obtained from many different tissues [14–16], Flt-1 but not KDR is highly expressed by trophoblast cells [17, 18]. Monocytes and peritoneal fluid macrophages respond to VEGF [19, 20], and melanomas have been shown to express VEGF receptors [21].

Angiogenesis is a complex process necessitating the interaction of numerous cell types that leads to the coordinated development of a complex three-dimensional vascular structure. Many factors are involved in angiogenesis, and it is the balance of stimulators such as VEGF and inhibitors like angiostatin that determines the net result [22, 23]. In the placenta there is profound angiogenesis as high-capacity transport develops between the maternal and fetal circulation. However, in the human, it is notable that little angiogenesis occurs in the maternal tissue while there is obvious capillary growth within the placental villi. This suggests that there are locally acting factors that regulate vascular growth.

We have previously shown that VEGF is highly expressed in the placenta and that early in gestation, maternal macrophages within the decidua contain large amounts of VEGF mRNA [17, 24]. We have also shown that villous and extravillous trophoblast (EVT) contain very high levels of mRNA encoding Flt-1 [17, 18]. In situ hybridization specific for Flt-1 shows a particularly strong signal over the villous and extravillous trophoblast cells with very little signal corresponding to the endothelial cells. The immunohistochemical localization of Flt-1, however, shows low-level uniform signal over each of these cell types. The probe used for the in situ hybridization detects both the membrane-bound and truncated, soluble forms of Flt-1 [25], while the antibody used in this study [18] reacts only with the C-terminus of the membrane-bound form. It is therefore possible that the trophoblast cells are producing a soluble form of Flt-1 lacking the cytoplasmic tyrosine kinase domain, which could act as a local antagonist for VEGF. We set out to determine whether soluble forms of Flt-1 were produced by the placenta and whether such molecules could play a role in regulating placental and decidual angiogenesis.

MATERIALS AND METHODS

Tissue and Serum Collection

The study was conducted in accordance with the conditions laid down by, and with the approval of, the ethical committee of Addenbrooke's Hospital (Cambridge, UK) N.H.S. Trust. Gestational dates were calculated from the first day of the last menstrual period and were ±7 days from those estimated at an ultrasound scan performed in the first trimester of pregnancy. First-trimester placental tissue was obtained after the surgical termination of pregnancy conducted between Weeks 8 and 12 of gestation, and third-trimester material was obtained from normal placentae at term after cesarean delivery. In the latter case, a thin

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[2]Correspondence: D. Stephen Charnock-Jones, Reproductive Molecular Research Group, Department of Obstetrics and Gynaecology, University of Cambridge, Box 223, The Rosie Hospital, Robinson Way, CB2 2SW, UK. FAX: 44 1223 215327; e-mail: dscj1@cam.ac.uk

slice of tissue was taken from the maternal surface of the placenta, which contained a significant amount of EVT and decidua, and also a 1-cm^3 block was excised from deeper in the placental cotyledon, containing only villous tissue. A sample from each area was checked by histology. Samples were either snap-frozen or fixed overnight in 10% buffered formalin and embedded in paraffin wax. Maternal serum samples were collected in nonheparinized tubes and after clotting and centrifugation were aliquoted and frozen.

Binding of ^{125}I-VEGF to Tissue Sections

Sections of frozen tissue were prepared, and binding assay of ^{125}I-VEGF was conducted according to the method of Shweiki et al. [26]. Sections were preincubated with 0.2% gelatin and 1 mg/ml heparin in PBS for 30 min at room temperature. ^{125}I-VEGF (Amersham International plc; 1500 Ci/mmol) was then added at a concentration of 100 pM in PBS. Serial control sections contained competitor VEGF (R&D Systems, Minneapolis, MN) at a concentration of 3 nM in the binding medium. After binding for 1 h at room temperature, sections were washed in cold PBS, fixed in 2.5% glutaraldehyde, and washed again. Sections were air dried, and autoradiographic emulsion (LM-1 emulsion; Amersham International plc, Buckinghamshire, UK) was applied to the slides, which were developed after 48 h.

Immunohistochemistry and Antibodies

Sections serial to those used for ^{125}I-VEGF binding were stained for CD34 to identify blood vessels. Briefly, sections were fixed for 5 min in 10% neutral buffered formalin, and anti-CD34 (Clone QBEND/10; Serotec, Raleigh, NC) was applied and detected with secondary biotin-streptavidin-horseradish peroxidase reagents from Zymed (Cambridge Biosciences, Cambridge, UK). Final detection was with diaminobenzidine. An anti-cytokeratin antibody (Clone MNF116; Dako Corp., Carpinteria, CA), as previously described, was used to identify epithelial cells and trophoblast [18]. There was no counterstaining of the tissue. In the Western blots, an anti-Flt-1 N-terminal antibody (fltNT) was used as described by Kendall et al. [27] and also a biotinylated goat polyclonal antibody directed against Flt-1 (BAF321; R&D Systems).

Cloning and Expression of Soluble Flt-1 (sFlt-1) from the Placenta

A cDNA library prepared in λgt11 from mRNA obtained from term placenta (Clontech, Palo Alto, CA) was screened using a probe specific for a portion of the extracellular domain of Flt-1. This probe (bases 1507 to 1779; Shibuya et al. [12]) is within an area showing minimal homology between Flt-1 and other related receptors and was obtained by polymerase chain reaction using the primers described by Charnock-Jones et al. [17]. Screening was performed as described in Charnock-Jones et al. [28]. Numerous hybridizing plaques were identified, and several of these were purified by further rounds of screening. Six of these clones, upon sequence analysis, proved to be similar to the clone described by Kendall and Thomas (EMBL accession number U01134 [25]) that encodes a soluble variant of Flt-1, termed sFlt-1 by us. The longest of these clones was fully sequenced using fluorescent dye terminators (ABI, The Perkin-Elmer Corp., Foster City, CA) and contained cDNA that encoded soluble Flt-1. The coding portion of this cDNA from the *Eag*I site at position 189 to the linker-derived *Eco*RI site at the 3′ end was subcloned into the baculoviral transfer vector pVL1392 (Pharmingen, San Diego, CA), and its orientation was confirmed by DNA sequencing. DNA was prepared from this plasmid using a Qiagen (Mississauga, ON, Canada) miniprep and then cotransfected into Sf9 cells with baculogold DNA (Pharmingen) to allow in vivo site-specific recombination to occur and the subsequent generation of recombinant viruses. The recombinant viruses were plaque purified in soft agar, and the plaques were identified by visual examination under strong illumination over a dark background. High-titer viral stocks were then prepared as described in the Pharmingen baculovirus manual. Protein expression was accomplished by infecting small-scale liquid cultures of approximately 100 ml of Sf9 cells at a density of approximately 2×10^6/ml with a high-titer viral stock at a multiplicity of infection of between 2 and 10. Cells were grown in complete TMN-FH medium (Pharmingen) or SF 900-II serum-free medium (Gibco/BRL, Gaithersburg, MD).

Protein Purification

Soluble receptor was purified using the method described by Kendall and Thomas [25]. Conditioned medium (45 ml) from the baculoviral-infected Sf9 cells was clarified and then passed through a 1-ml heparin-sepharose affinity column (Hi-trap column; Pharmacia Biotech, Milton Keynes, Bucks, UK) that had been previously equilibrated with 0.15 M NaCl in 10 mM sodium phosphate (pH 6.2). The column was washed with this buffer and also with the same buffer but containing 0.6 M NaCl. Soluble receptor was eluted from the column using 1.0 M NaCl in sodium phosphate buffer. The protein was desalted and concentrated further by centrifugal ultrafiltration (Centricon 30; Amicon Inc., Bedford, MA).

In Situ Hybridization

Primers specific for the novel 3′ end of sFlt-1 were 5′ AGAGGTGAGCACTGCAAC 3′ and 5′ ACTGCTATCATCTCCGAAC 3′. Use of these primers amplified a 215-base pair (bp) region of sFlt-1 from position 2215 to 2429 of the EMBL sequence (accession number U01134). Riboprobes were labeled with [^{33}P]UTP and in situ hybridization performed as described by Clark et al. [18].

RNase Protection Assay

The method used was essentially that of Charnock-Jones et al. [29]. An sFlt-1 probe was generated that corresponded to bp 2014 to 2297 of the sFlt-1 sequence (EMBL accession number U01134). This spans the region where sFlt-1 and the membrane-bound form differ, so that the full-length probe was protected for sFlt-1 while a smaller 205-bp fragment of RNA was protected for full-length Flt-1. The antisense probe was labeled with a total of 60 μCi of [α-^{32}P]UTP (800 Ci/mmol; Amersham International plc) using the MAXIscript in vitro transcription kit (Ambion, Austin, TX). The resulting probe was treated with RNase-free DNase to remove the template before being purified by preparative PAGE. For each 30-μg sample of placental RNA, 100 000 cpm of probe was mixed and precipitated before addition of the hybridization buffer (RPAII kit; Ambion). This mix was heat denatured and then hybridized overnight at 45°C. Excess probe was digested with RNase A, and the hybridized probe was ethanol precipitated and loaded onto a 6% polyacrylamide sequencing gel.

Placental RNA samples examined were from the surface and deep regions of term placenta and from the villi of first-trimester placenta collected as already described. Five samples from each group were examined. Results were expressed as a ratio of sFlt-1 to full-length Flt-1 mRNA.

Villus Culture

First-trimester villi from four patients were sorted and washed thoroughly with PBS. Each of the villus samples was cultured in a 60-mm Petri dish for 24 h; the medium was changed, and the samples were cultured for a further 48 h in 6 ml of serum-free, phenol red-free Dulbecco's Modified Eagle's medium (DMEM)/F12. Medium alone was subjected to the same conditions as a control. Samples of the villi were fixed for histological examination. Placental tissue obtained at term was cultured in a similar manner and purified as described above and by Kendall et al. [27].

Covalent Cross-Linking

^{125}I-VEGF (900 pM) was incubated overnight at room temperature with recombinant sFlt-1, trophoblast-conditioned medium, or heparin-sepharose affinity-purified serum. Control experiments were undertaken with 25 ng of VEGF (Amgen Inc., Thousand Oaks, CA), which competed with the ^{125}I-VEGF. Complexes were cross-linked for 5 min at room temperature with 5 mM *bis*(sulfosuccinimidyl)suberate (Pierce Chemical Company, Rockford, IL) dissolved in 5 mM sodium citrate (pH 5.0). The reaction was terminated with 1.0 M Tris-HCl (pH 6.8) containing 10% SDS and 8% glycerol. Samples were reduced by the addition of β-mercaptoethanol and subjected to electrophoresis on 4–15% gradient polyacrylamide gels (Bio-Rad, Cambridge, MA).

Inhibition of Binding of ^{125}I-VEGF to Bovine Aortic Endothelial (BAE) Cells

BAE cells, obtained from the laboratory of Dale Redmer of North Dakota State University (Fargo, ND) and cultured in DMEM/F12 with 10% fetal calf serum, were cultured to confluence in 24- or 48-well plates, and all binding experiments were conducted in triplicate at 4°C. Cells were rinsed in wash buffer (PBS containing 1 mg/ml BSA and 10^{-7} M KI). ^{125}I-VEGF at a final concentration of 20 pM was added to the binding medium (DMEM/F12; 25 mM Hepes; 1 mg/ml BSA; 10^{-7} M KI; 10 μg/ml heparin). To ascertain the level of nonspecific binding, identical incubations were performed in the presence of 2 nM unlabeled VEGF (R&D Systems). Cells were incubated for 2 h and rinsed three times with wash buffer before being solubilized in 0.5 M NaOH, and the levels of bound ^{125}I-VEGF were determined by gamma counting (Packard, Meriden, CT).

S-200 Gel-Filtration Chromatography

^{125}I-Labeled VEGF (Amersham International plc) was incubated at a final concentration of 15 pM with 400 μl of serum obtained from healthy male or female volunteers and from pregnant women at different stages of gestation. Samples of recombinant sFlt-1 and villus culture supernatant were also examined. After incubation overnight at room temperature, samples were analyzed by gel-filtration chromatography on a 12.5-ml S-200 Sephadex column (dimensions of 0.8 cm × 25 cm; Pharmacia and Upjohn, Kalamazoo, MI) as described by Hill et al. [30]. The column was equilibrated in PBS and eluted in the same buffer. Fractions (400 μl) were collected and the elution position of the ^{125}I-VEGF was determined by gamma counting (Packard). As controls, ^{125}I-VEGF was also run on the column in the absence of serum, as well as after incubation with serum and a large excess of unlabeled VEGF (5 nM).

^{125}I-VEGF Binding in Serum

Serum samples from nonpregnant women and from women in the third trimester of pregnancy were investigated (n = 3 in each group). Serum (5 ml) was mixed with an equal volume of 10 mM sodium phosphate (pH 6.2) with 0.15 M NaCl and passed through a 1-ml heparin-sepharose affinity column as described above for recombinant sFlt-1. The 0.6 M wash and 1.0 M eluted fractions were collected (6 ml of each), desalted, and concentrated 6-fold in Centricon 50 tubes (Amicon Inc.). Cross-linking and PAGE were conducted as described above.

RESULTS

Binding sites for VEGF within first-trimester and term placental tissue were investigated. Frozen tissue sections from a first-trimester (Week 10) placenta are representative of the localization pattern found at this stage of gestation (n = 3). Binding was present around the edge of the villi but not in a continuous layer. Areas of binding were detected in the villous core (Fig. 1, A and B). Serial or near-serial sections were immunostained with CD34 to detect blood vessels within the villous core (Fig. 1C). Comparison of CD34 staining and ^{125}I-VEGF localization confirmed that the strong binding was to the endothelial cells. ^{125}I-VEGF binding and immunostaining experiments for cytokeratin were conducted on the same sections (Fig. 1, D–F). No binding above background was detected over either of the villous trophoblast layers. This confirmed that the strong binding was to the blood vessels and not to the trophoblast cells. First-trimester decidual sections were also investigated, and a diffuse binding, above the levels of nonspecific controls, was observed (data not shown). This binding was specific and could be abolished by the addition of excess unlabeled VEGF.

On term placentae, ^{125}I-VEGF bound within large and small villi in a pattern compatible with localization to blood vessels (Fig. 2A; n = 3). A serial section immunostained for CD34 localized the blood vessels in the placenta; some light nonspecific staining was evident around the large villus in Figure 2B. Within the large villus (Fig. 2A), silver grains were particularly obvious in association with the blood vessels, in addition to there being some diffuse grains present within the stroma. A high-power photograph of a villus revealed that ^{125}I-VEGF binding was not over the trophoblast but within the villous core (Fig. 2C). In Figure 2D, a darkfield and a brightfield image have been merged. This clearly shows that the silver grains are associated with the blood vessels but also that there is some binding over the stroma. Over the decidua (Fig. 2E), diffuse but specific binding was detected. A serial control section to Figure 2E, which had the addition of 3 nM unlabeled VEGF in the binding mix, confirms that the diffuse silver grains in the decidua represent specific binding (Fig. 2F). The level of ^{125}I-VEGF binding in Figure 2F is representative of background levels found on control sections for Figures 1 and 2.

Cloning of sFlt-1 from the placenta was performed, and 6 of the 15 clones obtained from a placental cDNA library were sFlt-1 as opposed to full-length Flt-1. For sFlt-1, a 5′ sequence additional to that published by Kendall and Tho-

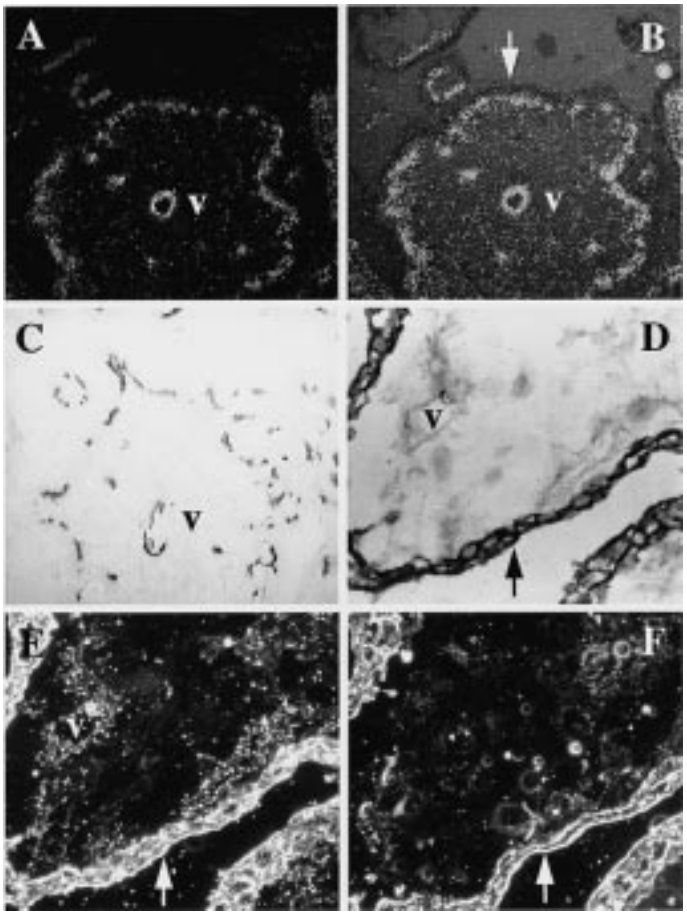

FIG. 1. ^{125}I-VEGF binding on first-trimester villus (Week 10). **A**) Darkfield image with specific binding in defined regions around a villus and associated with a blood vessel (v) within the villous core. **B**) Darkfield on brightfield of the same section as in **A**. Binding sites were not on the trophoblast layers (arrow) but just within it. Villous stroma had some low-level binding visible in both **A** and **B**. **C**) CD34 immunostaining for endothelial cells two sections away from **A** and **B**. Staining pattern mirrors that of the binding sites. **D–F**) A villus stained with an anti-cytokeratin antibody to identify the trophoblast (arrows) and also with ^{125}I-VEGF. This confirms that the binding is to the blood vessels (V) and not the trophoblast. **D**) Brightfield. **E**) Darkfield. **F**) Control ^{125}I-VEGF binding. **A–C**) ×85; **D–F**) ×330. Reproduced at 62%.

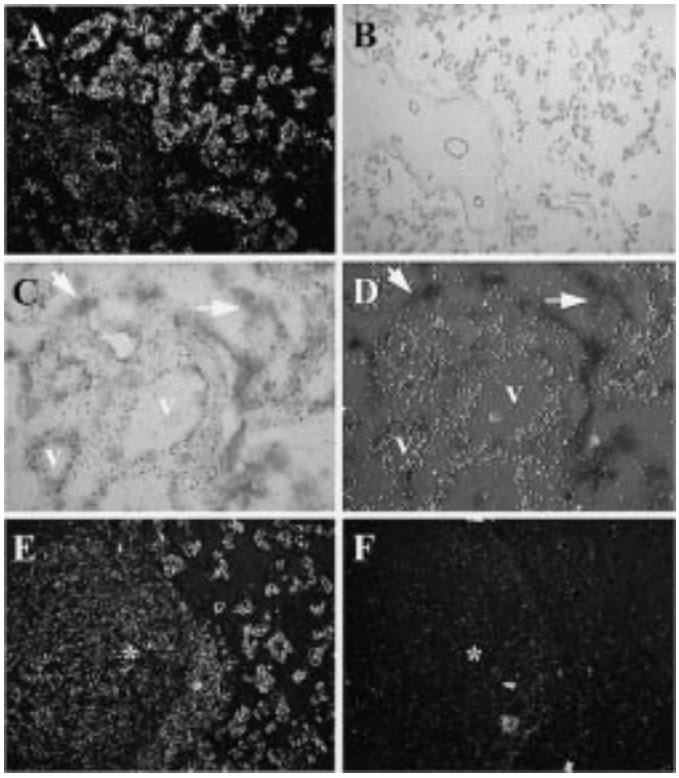

FIG. 2. ^{125}I-VEGF binding on tissue sections from term placenta. **A**) Darkfield photograph of binding within large and small villi in the placenta. **B**) CD34 immunostaining of blood vessels in a section serial to that in **A** reveals a similar localization pattern to that seen in **A**. **C** and **D**) Higher magnification with brightfield (**C**) and darkfield on brightfield (**D**) of the same villus. The trophoblast around the two villi (arrows) did not label, while the blood vessels (v) within the villi, and to a lesser extent the stromal core, bound ^{125}I-VEGF. **E** and **F**) Serial sections with darkfield and brightfield merged (**E**) and unlabeled VEGF as a competitor (**F**). In **E**, diffuse label present on decidua (asterisks) and within the villi (right side). Only diffuse background was present in **F**. **A, B, E, F**) ×80. **C, D**) ×330. Reproduced at 58%.

mas (EMBL accession number U01134) was obtained (5′ CGGAGCGCGCCAG 3′). At the 3′ end, a new poly A termination site was found. The translation termination site for sFlt-1 ends at bp 2313 in the Kendall and Thomas sequence, and the new poly A tail was found from bp 2331 onward.

In situ hybridization with a probe specific for sFlt-1 was used to confirm the presence of this mRNA in the placenta. Throughout pregnancy the EVT cells, which are present within the maternal decidual tissue, were highly positive for sFlt-1 (Fig. 3, A–D). Within the villous trophoblast, sFlt-1 mRNA was detected throughout pregnancy, but at varying levels both between placentae and within any one placenta (Fig. 3, E and F).

An RNase protection assay that could discriminate between mRNAs encoding sFlt-1 and Flt-1 was used to determine the relative levels of these mRNAs (data not shown). The tissues investigated included first-trimester villus and term placenta. In the term placenta, surface samples containing villus, decidua, and EVT, as well as deeper samples with only villi, were examined. Both Flt-1 forms were readily detectable, suggesting that there was a significant amount of mRNA present to encode these proteins. In all samples the full-length Flt-1 band was stronger than that for sFlt-1. The ratio of sFlt-1 to full length was approximately 0.45 in all three sampling areas.

The supernatants from placental villus cultures were examined to determine whether there was a soluble factor present that bound VEGF in a similar manner to recombinant sFlt-1. First-trimester villus was cultured in serum-free medium as described above, and the resulting medium was incubated with ^{125}I-VEGF overnight and cross-linked. Samples were reduced and examined after gel electrophoresis (Fig. 4). ^{125}I-VEGF bands, which could be displaced with unlabeled VEGF, were found at approximately 250 and 160 kDa. Villi were thus found to produce one or more soluble factors capable of binding VEGF. Recombinant sFlt-1 was also cross-linked with ^{125}I-VEGF, and this resulted in bands similar in molecular mass to those from the villus cultures (Fig. 4).

The binding of ^{125}I-VEGF to BAE cells was used to assay for the inhibitory activity of factors binding VEGF (Fig. 5). The heparin-sepharose affinity-purified recombinant sFlt-1 inhibited binding of ^{125}I-VEGF to the BAE cells. Supernatants from separate cultures of first-trimester villi were added to the VEGF binding assay at different concentrations. In all three cases the villus supernatants

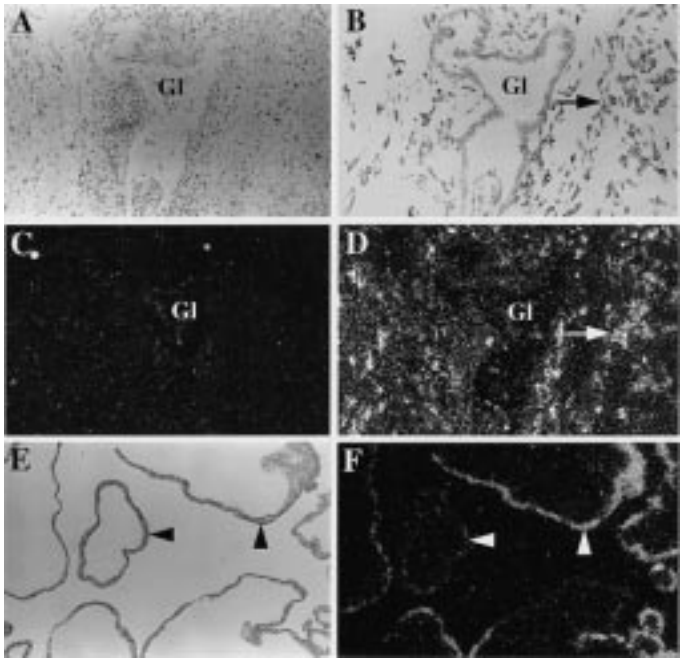

FIG. 3. In situ hybridization for sFlt-1. Serial sections from human placental tissue at Week 9 (**A–D**) and Week 14 (**E, F**) of gestation. **A**) Hematoxylin stain of maternal decidual tissue showing a large gland (Gl) and associated decidual tissue. **B**) Anti-cytokeratin antibody localization of the glandular epithelium and multiple EVT cells (arrow) within the maternal decidua. No counterstaining. **C**) Sense probe for sFlt-1. **D**) Antisense probe for sFlt-1. Probe evident over the EVT cells as indicated by the arrow. **E**) Anti-cytokeratin antibody, with no counterstaining, identifies the villous trophoblast cells (arrowhead). **F**) Antisense probe to sFlt-1. Varying levels of hybridization evident over the trophoblast layer (arrowhead). ×75. Reproduced at 48%.

showed dose-dependent inhibition of binding, although there was some variation in the activity of the culture supernatants.

Western blotting of the supernatants from villus cultures was undertaken (n = 4). Western blot analysis was performed using an anti-Flt-1 N-terminal antibody (FltNT) as described by Kendall et al. [27]. Figure 6A shows the presence of a soluble protein that reacts with the Flt-1 antibody and has a molecular mass of approximately 100 kDa. Villus-conditioned medium was purified by heparin-sepharose affinity and ion-exchange chromatography using the protocol described by Kendall et al. [27] for sFlt-1 (n = 2; Fig. 6B). This gave a band of slightly higher molecular mass to the recombinant sFlt-1. The recombinant sFlt-1 band was not detected when the antibody was preabsorbed with an excess of the peptide against which the antibody was raised. In a similar experiment, the band from the purified villus supernatant was not detected after preabsorption of the Flt-1 antibody with peptide. Using a polyclonal antibody raised against the extracellular domain of Flt-1 (BAF321; R&D Systems), a similar band could be readily detected in culture supernatants obtained using delivered placental material (Fig. 6C). In this instance the greater apparent difference in size compared to the recombinant sflt is due to the use of a different-strength polyacrylamide gel. In Figure 6C the resolution around 100 kDa has been maximized.

Since sFlt-1 was released into the villus supernatants, which in vivo would correspond to the maternal circulation, we investigated whether there were any VEGF-binding proteins that were unique to the serum of pregnant women. An

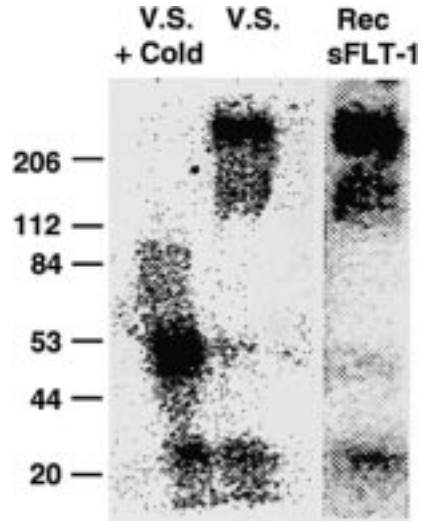

FIG. 4. Cross-linking of ^{125}I-VEGF to villus supernatant and recombinant sFlt-1. Villus supernatant (V.S.) and recombinant sFlt-1 (Rec sFlt-1) were each incubated and cross-linked with ^{125}I-VEGF, which resulted in a band at approximately 250 kDa and another weaker band at approximately 160 kDa. Unbound VEGF was present as a monomer and dimer at approximately 23 kDa and 46 kDa, respectively. Unlabeled VEGF added to the villus supernatant (V.S. + cold) competed for the high molecular weight binding.

S-200 Sephadex column was used to separate ^{125}I-VEGF and ^{125}I-VEGF complexes formed in samples of serum (n = 3 from each group), cultured villus supernatant (n = 3), and recombinant sFlt-1 (n = 2). ^{125}I-VEGF in PBS was analyzed in a similar manner to provide a baseline, and results showed that free VEGF was detected around fraction 30 with another small peak occurring around fraction 15. Binding at fraction 15 was found to occur consistently but

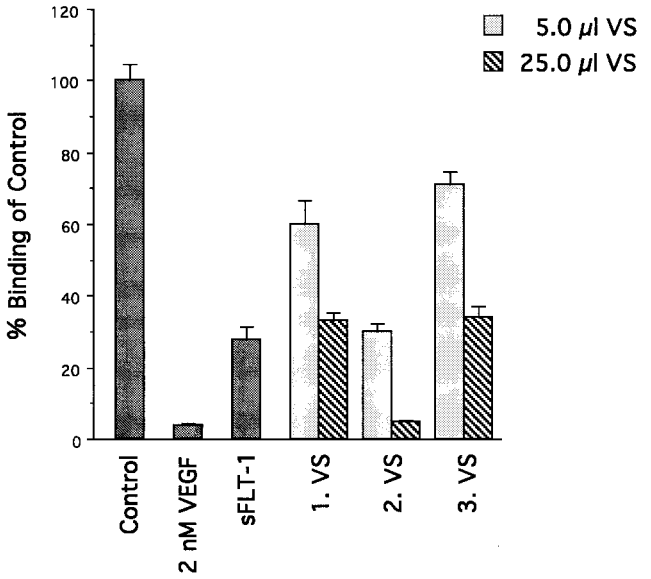

FIG. 5. Inhibition of ^{125}I-VEGF binding to BAE cells by villus supernatant. First-trimester villus from three individuals (1.VS to 3.VS) was cultured for 48 h in 6 ml of medium, and 5 μl or 25 μl of this supernatant was used in a 500-μl ^{125}I-VEGF binding assay conducted in the presence of heparin. Results are presented as the percentage of control. Unlabeled VEGF at 2 nM concentration almost entirely inhibited binding. Recombinant sFlt-1 partially inhibited binding. All three villus supernatants inhibited binding when added at 5 μl/well, and this was more pronounced when 25 μl was added. Error bars indicate SEM.

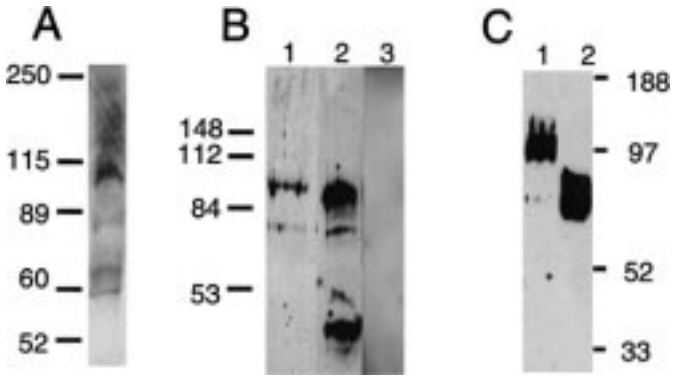

FIG. 6. **A–C**) Western blotting of villus supernatants using anti-N-terminal Flt-1 and anti-recombinant Flt-1 antibodies. First-trimester placental villi (**A** and **B**) and term placental tissue (**C**) were cultured and supernatants were collected, reduced, and run on a 4-15% gradient polyacrylamide gel. Western blotting was conducted with the anti-Flt-1 N-terminal antibody (fltNT, **A** and **B**) or BAF321 (**C**) (R&D Systems) and detected by enhanced chemiluminescence (Amersham). In the crude supernatant (**A**), an immunoreactive band was present at approximately 100 kDa, a size consistent with its being sFlt-1. In **B**, lane 1, villus supernatant that had been purified by heparin affinity and ion exchange, according to Kendall et al. [27], gave a band of the correct molecular mass for sFlt-1. Recombinant sFlt-1 was detected as seen in **B**, lane 2, and the immunoreactivity was absent after preabsorption of the antibody with the Flt-1 N-terminal peptide (lane 3). It is possible that the extra band detected in the recombinant sFlt-1 lane (**B**, lane 2) is an N-terminal fragment of sFlt-1. **B**, lane 1, contains 3.5 ng of recombinant sFlt-1 [27]; lane 2 contains a small fraction of the purified placental material. Detection was with the R&D antibody and enhanced chemiluminescence (Amersham).

was of a low affinity as it was competed for by other binding proteins (Fig. 7). Ligand blotting studies revealed that ^{125}I-VEGF binds to purified BSA (data not shown), and since the ^{125}I-VEGF is supplied with BSA as a carrier, it is probable that the binding seen at faction 15 is due to BSA. The three samples of male serum and nonpregnant female serum also gave a peak around fraction 15 as indicated by the arrow labeled BSA/VEGF (Fig. 7, male). In serum samples from early pregnancy (Week 12) and at term, ^{125}I-VEGF bound to a larger serum protein, and this was eluted from the column around fractions 10–11 (Fig. 7, arrow). This binding competed with the BSA/VEGF peak. At term, one of the three samples had very high levels of binding in this fraction. Samples from cultured first-trimester villus supernatant gave a peak at fractions 10–11, and recombinant sFlt-1 gave a peak at fraction 11. Control medium (DMEM/F12) for the villus culture experiments gave only a small peak at fraction 15.

^{125}I-VEGF binding was conducted in serum samples that had been purified by heparin affinity (according to Kendall and Thomas [25]). In all three serum samples from pregnant women, the 1.0 M NaCl fraction contained a VEGF-binding protein that when complexed with ^{125}I-VEGF gave a molecular mass just over 250 kDa (Fig. 8, lane 1). This protein was not present in the nonpregnant samples (lane 2), and binding to it could be competed for with unlabeled VEGF (lane 3). The control 0.6 M fraction from pregnant serum had a small amount of bound ^{125}I-VEGF at the same molecular mass as the 1.0 M fraction (lane 4). It is likely that this is the same binding protein and that a small amount was eluted from the column in the washes. No binding was evident with the 0.6 M fraction from nonpregnant samples (lane 5).

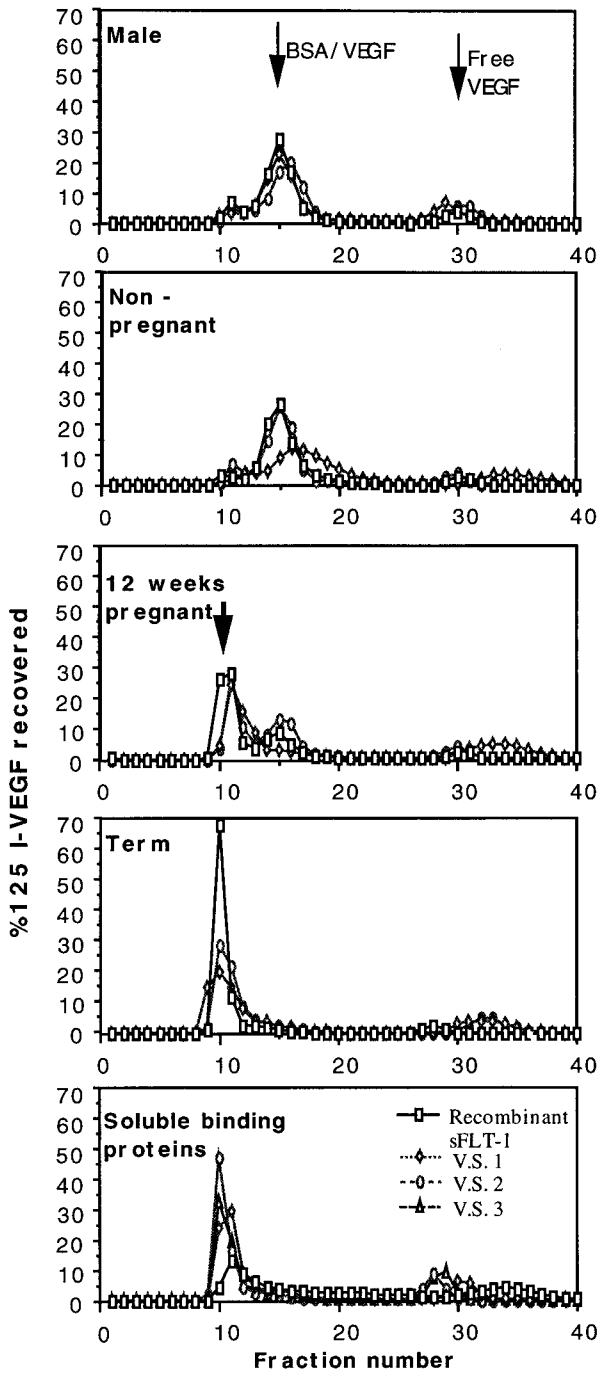

FIG. 7. S-200 gel-filtration chromatography. Each graph presents data from three different individuals. The arrow labeled BSA/VEGF shows a competitive peak at around fraction 15. Free VEGF came off at the fractions marked by an arrow. In samples from Week 12 of pregnancy and those from term, a peak was detected at fractions 10-11 (arrow). Likewise, in samples of villus supernatant (V.S.) and recombinant sFlt-1, a peak was present at fractions 10-11.

DISCUSSION

Previous research has investigated the production and localization of VEGF and its receptors in the human placenta [18, 31–33]. A discrepancy between the immunohistochemical localization and in situ hybridization patterns observed for Flt-1 by Clark et al. [18] suggested that the villous trophoblast may produce a soluble receptor to VEGF. This report demonstrates that sFlt-1 is produced by

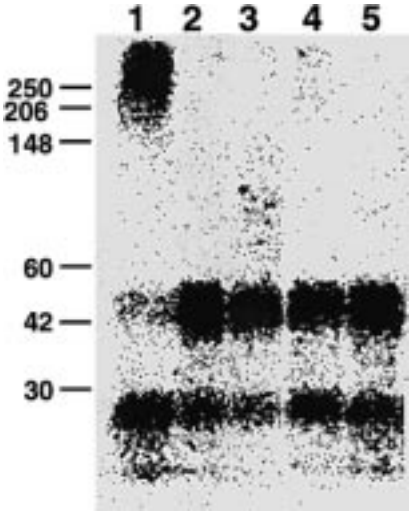

FIG. 8. Cross-linking of human serum with ^{125}I-VEGF and gel electrophoresis. Serum from nonpregnant women and pregnant women at term was placed on a heparin column, and the 0.6 M wash and 1.0 M NaCl fractions were collected. Samples were incubated with ^{125}I-VEGF and cross-linked before PAGE. Unbound VEGF was detected as both a monomer and dimer at 23 and 46 kDa, respectively. Lane 1) 1.0 M NaCl fraction of serum from term pregnant women with bound ^{125}I-VEGF at a molecular mass just over 250 kDa. Lane 2) 1.0 M NaCl fraction of serum from nonpregnant women with only unbound VEGF. Lane 3) Unlabeled VEGF competed for the band observed with the 1.0 M fraction from term pregnant women in lane 1. Lane 4) 0.6 M NaCl fraction from the serum of term pregnant women with only a small amount of bound VEGF just over 250 kDa. Lane 5) 0.6 M NaCl fraction from the serum of nonpregnant women with no bound VEGF.

both villous and extravillous trophoblast. A rare mRNA encoding sFlt-1 was previously cloned from human umbilical vein endothelial cells (HUVEC) by Kendall and Thomas [25] and purified from HUVEC culture media [27]. This is, however, the first report of its production in vivo, with implications for VEGF-mediated angiogenesis and placental function.

Ligand binding was performed on first- and third-trimester human placental sections. ^{125}I-VEGF was found to principally bind to the fetal blood vessels within the villi (Figs. 1 and 2). Under the conditions used it is likely that the ^{125}I-VEGF would have bound to both Flt-1 and KDR. Although estimates of the K_d values for these receptors of VEGF vary dramatically, there does seem to be a consensus that the Flt-1 high-affinity sites have a dissociation constant of between 1 and 16 pM and that the lower-affinity KDR sites are between 10 and 100 times that of Flt-1 [16, 34–36]. Both Flt-1 and KDR-like immunoreactivities have been localized to the fetal endothelial cells of the placental villi throughout pregnancy [18, 33], and the binding sites reported here confirm these results and show that fetal endothelial cells have specific high-affinity receptors for VEGF. This is in agreement with what is widely found in other tissues [37].

When investigating the localization of Flt-1 mRNA in the maternal decidual tissue, we previously used an in situ hybridization probe specific for the extracellular domain of Flt-1 and detected very strong signal over the EVT cells throughout pregnancy [17, 18]. Immunohistochemical localization with antibodies directed against the intracellular carboxyl terminus of Flt-1 indicated that some of the Flt-1 produced by these cells may be full-length membrane-bound receptor [18, 31, 32]. However, a striking feature of the ^{125}I-VEGF binding studies is the lack of detectable binding in association with the EVT or any other specific cell within the decidua. Some diffuse but specific binding, which could be displaced by excess unlabeled VEGF, was detected over the maternal decidual tissue. It is possible that some ^{125}I-VEGF associates with a soluble VEGF receptor that then interacts with the heparan sulfates present in the extracellular matrix. The in situ hybridization described in this study using a probe specific for sFlt-1 revealed that EVT cells contain large amounts of mRNA encoding this soluble VEGF receptor. It is therefore unlikely that VEGF will have any stimulatory effect on this population of cells. It is also unlikely that VEGF has appreciable effects on villous trophoblast in vivo since there is no specific binding of ^{125}I-VEGF to these cells and they also produce soluble Flt-1.

The decidua is a site where large amounts of VEGF are produced by the macrophages in Nitabuch's stria during the first trimester of pregnancy [17] but where the process of vascular transformation is required rather than angiogenesis. A pool of sFlt-1 in the decidua may therefore be important for reducing the effects of VEGF on maternal endothelial cells within this tissue.

Significant quantities of both full-length Flt-1 and sFlt-1 mRNA were present in the placenta and were readily detectable by RNase protection assays, although there was more mRNA encoding full-length Flt-1. As yet it is not known whether there are differences in the half-lives of these two species or whether the ratio observed for the mRNA is reflected in the protein levels. The Flt-1 mRNAs identified to date arise by alternative splicing [25]. Understanding the mechanisms by which this splicing is controlled will have important consequences for the biological actions of VEGF and hence angiogenesis.

In situ hybridization using a probe specific for the novel 3′ terminus of sFlt-1 revealed that the mRNA for sFlt-1 was present within villous trophoblast throughout pregnancy, although, interestingly, there was variability in the intensity within and between placentae. Analysis by an RNase protection assay of superficial and deep samples of placenta (i.e., tissue samples with and without substantial quantities of EVT cells present) showed that the ratio of Flt-1 to sFlt-1 mRNA remained the same, indicating that the villous trophoblast is a significant source of sFlt-1 mRNA throughout pregnancy. Because the size of the placenta, and thus the quantity of villous trophoblast, increase dramatically during pregnancy, it is likely that there will be an increase in the total sFlt-1 production.

To determine whether the placenta is capable of secreting a protein with the characteristics of a soluble VEGF receptor, first-trimester villi and placental tissue obtained at delivery were cultured in serum-free media and the supernatants analyzed. Results from the gel-filtration chromatography, cross-linking, and the BAE binding assay were consistent with the presence of biologically active soluble receptor (Figs. 4, 5, and 7). To conclusively demonstrate the presence of sFlt-1, an antibody to the N-terminus of Flt-1 or one raised against the entire extracellular domain of Flt-1 was used. Western blotting with both Flt-1 antibodies identified a soluble protein in the villus supernatants (Fig. 6). Furthermore, a protein could be purified using the same conditions as for recombinant sFlt-1, and this protein was immunoreactive with two Flt-1 antibodies and was of a molecular mass consistent with its being sFlt-1 [25, 27].

Recombinant forms of sFlt-1 have been shown to inhibit the action of VEGF in vitro [25], and, furthermore, artificially engineered VEGF-receptor chimeric proteins have

been shown to block VEGF-induced retinal neovascularization [38]. This indicates that soluble VEGF-binding proteins can play a role in the regulation of VEGF action in vivo.

In the placenta, the villi are in direct contact with maternal blood. Proteins secreted into the supernatant in vitro may therefore be detectable in maternal circulation. We investigated whether VEGF-binding activity could be identified in the serum of pregnant women. Using the gel-filtration assay described above, serum from women in the first trimester of pregnancy and at term had binding activity that was not detectable in the serum from nonpregnant women or males (Fig. 7). Consistent with this activity's being sFlt-1 was the purification of a binding protein, using the method for purifying sFlt-1 [25], from the serum of pregnant women at term that was not detected in serum from nonpregnant women (Fig. 8). The presence of a VEGF-binding protein in serum has implications for regulating the levels of bioavailable VEGF during pregnancy [39]. Although the placenta produces sFlt-1 and is thus likely to be an important source of this protein, there is the possibility that sFlt-1 production may be up-regulated in other organs during pregnancy.

It has been suggested that α2-macroglobulin, present in high levels in human serum, binds and inactivates VEGF [40]; however, the column fractionation and cross-linking studies conducted here did not detect this binding in any of the serum samples tested (male; nonpregnant and pregnant female). In addition, after column fractionation of the serum, the binding fractions were immunoblotted and no immunoreactivity was found with an antibody against α2-macroglobulin.

The balance between positive and negative effectors in the regulation of angiogenesis is now recognized as having considerable importance [23]. Thus, agents that specifically antagonize the actions of VEGF are of great interest. It is not currently known whether any other tissues produce significant amounts of sFlt-1. In the serum samples from nonpregnant women and males, no significant VEGF-binding activity could be detected. If additional tissues do produce VEGF-binding proteins, then their contribution to serum levels, at least in normal subjects, is significantly lower than that seen during pregnancy. We have found mRNA encoding sFlt-1, by reverse transcription-polymerase chain reaction, in tissue samples from the kidney, renal vein, adrenal, aorta, and pulmonary artery (data not shown). The possibility thus exists that sFlt-1 may locally mediate the action of VEGF in various tissues. Of considerable interest would be whether the production of either locally acting or circulating soluble receptors for VEGF are associated with various pathological conditions.

In conclusion, we have shown that the human placenta is a rich source of a soluble VEGF receptor that antagonizes the action of VEGF. This receptor has been characterized and shown to be sFlt-1, which had previously been detected only at low levels in cultured HUVEC [27]. This protein is present in maternal serum and so may influence the maintenance functions of VEGF on endothelial cells [4] in addition to blocking angiogenesis locally in the decidua.

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REFERENCES

1. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol 1991; 5:1806–1814.
2. Charnock-Jones DS, Sharkey AM, Rajput-Williams J, Burch D, Schofield PJ, Fountain SA, Boocock CA, Smith SK. Identification and localization of alternately spliced mRNAs for vascular endothelial growth factor in human uterus and steroid regulation in endometrial carcinoma cell lines. Biol Reprod 1993; 48:1120–1128.
3. Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumour vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest 1989; 84:1470–1478.
4. Alon T, Hemo I, Itin A, Pe'er J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med 1995; 1:1024–1028.
5. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, Ferrara N. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature 1993; 362:841–844.
6. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A. Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. Nature 1994; 367:576–579.
7. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea SK, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; 380:439–442.
8. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380:435–439.
9. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. EMBO J 1996; 15:290–298.
10. Olofsson B, Pajusola K, Kaipainen A, von Euler G, Joukov V, Saksela O, Orpana A, Pettersson RF, Alitalo K, Eriksson U. Vascular endothelial growth factor B, a novel growth factor for endothelial cells. Proc Natl Acad Sci USA 1996; 93:2576–2581.
11. Conn G, Bayne ML, Soderman DD, Kwok PW, Sullivan KA, Palisi TM, Hope DA, Thomas KA. 1 Amino acid and cDNA sequences of a vacular endothelial cell mitogen that is homologous to platelet-derived growth factor. Proc Natl Acad Sci USA 990; 87:2628–2632.
12. Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene 1990; 5:519–524.
13. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, Bohlen P. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. Biochem Biophys Res Commun 1992; 187:1579–1586.
14. Peters KG, de Vries C, Williams LT. Vascular endothelial growth factor receptor expression during embryogenesis and tissue repair suggest a role in endothelial differentiation and blood vessel growth. Proc Natl Acad Sci USA 1993; 90:8915–8919.
15. Millauer B, Wizigmann-Voos S, Schnurch H, Martinez R, Moller NPH, Risau W, Ullrich A. High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis. Cell 1993; 72:835–846.
16. Olander JV, Connolly DT, DeLarco JE. Specific binding of vascular permeability factor to endothelial cells. Biochem Biophys Res Commun 1991; 175:68–76.
17. Charnock-Jones DS, Sharkey AM, Boocock CA, Ahmed A, Plevin R, Ferrara N, Smith SK. Vascular endothelial growth factor receptor localisation and activation in human trophoblast and choriocarcinoma cells. Biol Reprod 1994; 51:524–530.
18. Clark DE, Smith SK, Sharkey AM, Charnock-Jones DS. Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy. Hum Reprod 1996; 11:1090–1098.
19. Clauss M, Gerlach M, Gerlach H, Brett J, Wang F, Familletti PC, Pan Y-CE, Olander JV, Connolly DT, Stern D. Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration. J Exp Med 1990; 172:1535–1545.

20. McLaren J, Prentice A, Charnock-Jones DS, Millican SA, Muller KH, Sharkey AM, Smith SK. Vascular endothelial growth factor is produced by peritoneal fluid macrophages in endometriosis and is regulated by ovarian steroids. J Clin Invest 1996; 98:482–489.
21. Potgens AJG, Lubsen NH, van Altena MC, Schoenmakers JGG, Ruiter DJ, de Waal RMW. Vascular permeability factor expression influences tumor angiogenesis in human melanoma lines xenografted to nude mice. Am J Pathol 1995; 146:197–209.
22. O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by Lewis lung carcinoma. Cell 1994; 79:315–328.
23. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996; 86:353–364.
24. Sharkey AM, Charnock-Jones DS, Boocock CA, Brown KD, Smith SK. Expression of mRNA for vascular endothelial growth factor in human placenta. J Reprod Fertil 1994; 99:609–615.
25. Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci USA 1993; 90:10705–10709.
26. Shweiki D, Itin A, Neufeld G, Gitay-Goren H, Keshet E. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. J Clin Invest 1993; 91:2235–2243.
27. Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, Flt-1, and its heterodimerization with KDR. Biochem Biophys Res Commun 1996; 226:324–328.
28. Charnock-Jones DS, Day K, Smith SK. Cloning, expression and genomic organisation of human placental protein disulfide isomerase. Int J Biochem Cell Biol 1995; 28:81–89.
29. Charnock-Jones DS, Sharkey AM, Fenwick P, Smith SK. Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. J Reprod Fertil 1994; 101:421–426.
30. Hill DJ, Tevaarwerk GJM, Arany E, Kilkenny D, Gregory M, Langford KS, Miell J. Fibroblast growth factor-2 (FGF-2) is present in maternal and cord serum, and in the mother is associated with a binding protein immunologically related to the FGF receptor-1. J Clin Endocrinol Metab 1995; 80:1822–1831.
31. Cooper JC, Sharkey AM, McLaren J, Charnock-Jones DS, Smith SK. Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry. J Reprod Fertil 1995; 105:205–213.
32. Ahmed A, Li XF, Dunk C, Whittle MJ, Rushton DI, Rollason T. Colocalisation of vascular endothelial growth factor and its Flt-1 receptor in human placenta. Growth Factors 1995; 12:235–243.
33. Vuckovic M, Ponting J, Terman BI, Niketic V, Seif MW, Kumar S. Expression of the vascular endothelial growth factor receptor, KDR, in human placenta. J Anat 1996; 188:361–366.
34. Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin C-H. Different signal transduction properties of KDR and Flt-1, two receptors for vascular endothelial growth factor. J Biol Chem 1994; 269:26988–26995.
35. Seetharam L, Gotoh N, Maru Y, Neufeld G, Yamaguchi S, Shibuya M. A unique signal transduction from flt tyrosine kinase, a receptor for vascular endothelial growth factor VEGF. Oncogene 1995; 10: 135–147.
36. Vaisman N, Gospodarowicz D, Neufeld G. Characterisation of the receptors for vascular endothelial growth factor. J Biol Chem 1990; 265:19461–19466.
37. Jakeman LB, Armanini M, Phillips HS, Ferrara N. Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis. Endocrinology 1993; 133:848–859.
38. Aiello LP, Pierce EA, Foley ED, Takagi H, Chen H, Riddle L, Ferrara N, King GL, Smith LEH. Suppression of retinal neovascularization in vivo by inhibition of vascular endothelial growth factor (VEGF) using soluble VEGF-receptor chimeric proteins. Proc Natl Acad Sci USA 1995; 92:10457–10461.
39. Sharkey AM, Cooper JC, Balmforth JR, McLaren J, Clark DE, Charnock-Jones DS, Morris NH, Smith SK. Maternal plasma levels of vascular endothelial growth factor in normotensive pregnancies and in pregnancies complicated by pre-eclampsia. Eur J Clin Invest 1996; 26:1182–1185.
40. Soker S, Svajm CM, Neufeld G. Vascular endothelial growth factor is inactivated by binding to α2-macroglobulin and the binding is inhibited by heparin. J Biol Chem 1993; 268:7685–7691.