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Localization of VEGF and expression of its receptors flt and KDR in human placenta throughout pregnancy

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Vascular endothelial growth factor (VEGF) is a potent secreted angiogenic growth factor. Its action is mediated through the tyrosine kinase receptors flt and KDR. We here examine, in detail, the distribution of this ligand and its receptors in human placentae throughout gestation. In the first trimester, in-situ hybridization revealed uneven distribution of flt mRNA around the villous trophoblast indicating spatial regulation. Temporal regulation of flt was observed with no flt mRNA expression detected in villi from mid-gestational placenta, while low levels were found in term villi. Extravillous trophoblast was found to contain both mRNA encoding flt and flt-like immunoreactivity throughout pregnancy. In contrast, KDR mRNA was found only in association with endothelial cells. Within the decidua the anti-flt antibody stained multiple cell types during the first trimester of pregnancy but only the extravillous trophoblast later in gestation. VEGF immunoreactivity tended to co-localize with the staining for flt. These results indicate that VEGF may exert an important role within both the placental villi and the maternal decidua in relation to the growth, differentiation and migration of trophoblast and that this is mediated primarily through the spatial and temporal regulation of the flt receptor rather than the KDR receptor.

Key words: decidua/endothelial/trophoblast/tyrosine kinase/uterus

Introduction

Placental function is inextricably linked with the vascular physiology of both fetal and maternal circulations found within it. During pregnancy the exchange capacity across the placental circulations must increase to accommodate the demands of the growing fetus. This is achieved by growth of the villous tree as well as alterations to the villous trophoblast lining the maternal blood space and the fetal blood vessels within the villi. To ensure sufficient blood flow through the placental bed the invasive extravillous trophoblast penetrates through the decidualized endometrium and modifies the maternal spiral arteries leading to their transformation into high flow, low resistance vessels. In addition a great number of extravillous trophoblast cells are found within the maternal placental tissue but not in association with maternal blood vessels; the physiological importance of such trophoblast is largely unknown (Benirschke and Kaufmann, 1995).

Vascular endothelial growth factor (VEGF) or vascular permeability factor (VPF) is a secreted dimeric glycoprotein with angiogenic properties (Connolly *et al.*, 1989; Ferrara *et al.*, 1992). To date, five splice variants of 121, 145, 165, 189 and 206 amino acids have been identified in human tissue (Houck *et al.*, 1991; Charnock-Jones *et al.*, 1993). Its biological activities include induction of vascular permeability (Connolly *et al.*, 1989; Keck *et al.*, 1989; Dvorak *et al.*, 1995), stimulation of endothelial cell division and migration (Dvorak *et al.*, 1995; Grimwood *et al.*, 1995) and in-vivo angiogenesis (Connolly *et al.*, 1989). As well as being angiogenic, VEGF also has a role in the maintenance of newly-formed blood capillaries (Alon *et al.*, 1995).

VEGF has two known receptors, the fms-like tyrosine kinase (flt) and the kinase insert domain-containing receptor (KDR) (de Vries *et al.*, 1992; Terman *et al.*, 1992). There are several soluble variants of the flt receptor (Kendall and Thomas, 1993; Boocock *et al.*, 1995). Membrane-bound flt binds VEGF with higher affinity than KDR (Waltenberger *et al.*, 1994).

The importance of these receptors in relation to endothelial cell biology can be deduced from the generation of null mutations in the equivalent mouse genes. Inactivation of the mouse *flk-1* gene (equivalent to human KDR) is associated with a failure in endothelial cell development and means that no organized vascular system forms (Shalaby *et al.*, 1995). The null mutation of the *flt* gene, like that for *flk-1*, results in fetal death but is associated with a different type of endothelial cell defect than observed for KDR. In the *flt*-deficient mice the endothelial cells differentiate but are unable to form correct vascular structures, possibly because of incorrect positional or environment cues (Fong *et al.*, 1995).

Some information is available regarding the distribution of VEGF in the placenta but there are no complete data describing its localization throughout pregnancy within both villi and decidua. During the first trimester of pregnancy VEGF mRNA has been only weakly detected in cytotrophoblast, syncytiotrophoblast and Hofbauer cells of the villi. However, a strong hybridization signal has been found over the macrophages within Nitabuch's layer and in maternal glandular epithelium (Sharkey *et al.*, 1993). Jackson *et al.* (1994) found immunoreactive VEGF on cytotrophoblast but not on Hofbauer cells whilst Cooper *et al.* (1995) found localization on Hofbauer cells but not cytotrophoblast. During the second trimester VEGF immunoreactivity was present on syncytiotrophoblast but this staining was only light (Jackson *et al.*, 1994). At the time of

parturition, VEGF mRNA and protein have been localized to the syncytiotrophoblast (Jackson *et al.*, 1994), to the extravillous trophoblast and to Hofbauer cells (Sharkey *et al.*, 1993; Cooper *et al.*, 1995). Immunohistochemical staining of extracellular matrix within the villous core has also been noted (Jackson *et al.*, 1994; Cooper *et al.*, 1995).

The localization of flt and KDR within the placenta has not been investigated in detail. Barleon *et al.* (1994), using Northern blotting, detected only negligible expression of the KDR gene in relation to the presence of very high levels of the flt gene. Localization of flt mRNA indicated that in the first trimester the villous cytotrophoblast produced low amounts but that the extravillous trophoblast and the base of cytotrophoblast columns produced the most. At term flt was only very weakly expressed within the trophoblast of the villi but strongly within the extravillous trophoblast (Charnock-Jones *et al.*, 1994).

Here we present a detailed overview of the localization of VEGF and its receptors flt and KDR in the human placenta throughout gestation.

Materials and methods

Tissue collection and processing

The study was conducted in accordance with approval given by the ethical committee of the Addenbrookes' Hospital NHS Trust, Cambridge, UK. Gestational dates of human placentae used in this study were calculated from the 1st day of the last menstrual period and were within ± 7 days of an ultrasound scan performed in the first trimester of pregnancy. First and third trimester material was obtained from normal placentae. Placentae used for second trimester analysis were from fetuses with anencephaly. All procedures were conducted on tissue sections from formalin-fixed, paraffin embedded, tissue blocks. Sections (4 μm) were cut onto slides coated with 3-aminopropyltriethoxy-silane (Sigma Chemical, Poole, UK). Immuno- and in-situ localization procedures were conducted on serial sections from the same placenta. Numbers of placentae examined at each stage of gestation are presented in Table I.

Table I. Gestational dates of placenta used in this study

	Immunohistochemistry	In-situ hybridization
First trimester	Week 8 (×2)*	Week 8 (×2)*
	Week 9*	Week 9 (×2)*
		Week 10
Second trimester	Week 14	Week 14
	Week 18	Week 18
	Week 19	Week 19
	Week 22	Week 22
Third trimester	Term (×3)	Term (×2)
		flt has 2 extras

*Matched set of decidual and villus tissue from each patient.

Immunohistochemistry

VEGF was detected using an antipeptide antibody directed against the amino terminus of the human protein (Santa Cruz Biotechnology Inc., Wiltshire, UK). A goat anti-hVEGF (R & D Systems, Oxon, UK) was also investigated for use on paraffin sections and gave similar results. The antipeptide antibody against flt detected a region at the carboxyl terminus of the molecule (Santa Cruz Biotechnology). Trophoblast cells were identified with a monoclonal antibody against cytokeratin (Clone MNF116; Dako Ltd., High Wycombe, UK). The mouse anti-human CD 68 (Clone PG-M1; Dako) was used to identify monocytes and macrophages.

Controls for the immunohistochemistry included substituting the equivalent amount of rabbit immunoglobulin (Ig)G (Dako) in place of the primary antibody as well as absorption of the antibody with a 20× access of the control peptides for flt and VEGF (Santa Cruz Biotechnology).

Tissue sections were dewaxed in xylene and hydrated through ethanol to water. Those sections to be stained for flt were incubated in 6 M urea for 30 min prior to pressure cooking. To unmask the tissue antigens for VEGF, flt and cytokeratin, sections were briefly treated in a pressure cooker (Sigma method). To perform this, 1500 ml of 0.01 M sodium citrate buffer was brought to boiling point in a Prestige pressure cooker. Slides in metal racks were submerged in the buffer and the lid sealed on the pressure cooker. Once at pressure the sections were left for exactly 1 min then the pressure valve released and the cooker cooled by placing under cold running water. The rack of slides was removed immediately and washed for 5 min (×2) in phosphate-buffered saline (PBS). Pre-treatment for CD-68 immunostaining was with 0.1% protease (Code No. P8038; Sigma) in 0.1% $CaCl_2$ buffer at room temperature for 10 min.

Following the pre-treatment, slides were incubated in PBS, 1% bovine serum albumin (BSA) with 20% heat inactivated goat serum (Sigma) for 30 min at room temperature. VEGF and flt antibodies were used at a concentration of 1 μg/ml, anti-cytokeratin at 9 μg/ml and anti-CD68 at 36 μg/ml in PBS, 1% BSA, 5% goat serum. Sections were incubated overnight, at 4°C, in the presence of the primary antibody. Slides were washed (three changes over 1 h) with PBS, 0.1% Tween-20 before the application of the secondary biotinylated antibodies of either goat anti-rabbit at 1:300 dilution for sections with polyclonal antibodies or goat anti-mouse at 1:500 dilution for the monoclonal antibodies (Dako). The secondary antibody was incubated for 1 h at room temperature before being washed for 15 min with three changes of PBS containing 0.1% Tween-20. Endogenous peroxidases within the tissue were quenched for 10 min with 0.3% H_2O_2 in methanol. Washing for 3×5 min with PBS and application of the avidin–biotinylated horseradish peroxidase complex (Vectastain Elite ABC kit; Vector Lab., Peterborough, UK) was followed by detection with diamino-benzidine (Sigma). In most sections Mayer's haemalum counterstaining was undertaken. Sections were photographed using a Leica DMRB/E microscope with a 35 mm camera attachment.

In-situ hybridization

The in-situ hybridization protocol used in this study was based on the methods of Tisdall *et al.* (1994) and Braw-Tal *et al.* (1994).

Section preparation

Sections were dewaxed then immersed in 0.2 N HCl for 20 min at room temperature and washed in 2× sodium chloride/sodium citrate (SSC) (1× SSC contains 150 mM sodium chloride and 15 mM sodium citrate, pH 7.0) for 30 min. Proteinase K (Sigma) digestion was undertaken at a concentration of 2 μg/ml in 200 mM Tris-HCl (pH 7.2), 50 mM EDTA (pH 8.0) at 37°C for 15 min. The slides were then immersed for 2×5 min at room temperature in a solution of 100 mM triethanolamine (pH 8.0), 0.25% acetic anhydride. Slides were washed in 2× SSC at room temperature for 5 min, dehydrated and dried.

Probe preparation

The flt and KDR probes have been previously described by Boocock *et al.* (1995). Briefly, the flt cDNA probe of 273 bps corresponds to

bases 1507–1779, when numbering from the ATG as presented by Shibuya *et al.* (1990). The KDR cDNA corresponds to bases 1360–1764 from the initiation codon (Terman *et al.*, 1992) giving a 405 bp probe. Both probes were from regions encoding the extracellular domain and showed considerable divergence thus ensuring specific hybridization.

The transcription vector Bluescript II KS (Stratagene Ltd., Cambridge, UK) which contained the flt and KDR templates was linearized with appropriate restriction enzymes. Single stranded sense and antisense RNA probes, with [^{33}P]-UTP label, were transcribed (MAXIscript in vitro transcription kit, Ambion; AMS Ltd., Witney, UK).

Hybridization

Riboprobe (at ~2×10^6 c.p.m./μl) was mixed with 20–60 μl of hybridization buffer. The hybridization buffer contained: 50% (v/v) deionized formamide, 0.3 M NaCl, 10 mM Tris-HCl pH 6.8, 10 mM sodium phosphate pH 6.8, 5 mM EDTA pH 8.0, 1× Denhardts solution (0.02 % (w/v) each of BSA, Ficoll and polyvinyl pyrrolidone), 10% (w/v) dextran sulphate, 50 mM DTT (dithiothreitol) and yeast tRNA 1 mg/ml. This mixture, containing the probe, was denatured at 95°C and applied to the pretreated and dried tissue sections which were then covered with small pieces of parafilm. Hybridization was performed for 18 h at 55°C in a sealed container humidified with 50% formamide and 0.3 M NaCl.

The slides were washed in 5× SSC at 50°C for 15 min (×2) and then in 2× SSC, 50% formamide at 65°C for 30 min. Four washes, of 5 min duration, were conducted in 2× SSC at 37°C. Slides were incubated at 37°C for 30 min with RNase A (Sigma) at a final concentration of 20 μg/ml in 1× wash solution (400 mM NaCl, 10 mM Tris-HCl pH 7.5, 5 mM EDTA). The RNase A was removed by washing the slides in 2× SSC, 50% formamide at 65°C for 30 min followed by 15 min washes in 2× SSC and 0.2× SSC, both at 37°C. Sections were dehydrated through 30, 60, 80 and 95% ethanols containing 0.3 M ammonium acetate then two final washes in 100% ethanol alone. Sections were air-dried and coated with autoradiographic emulsion (LM-1 emulsion; Amersham International plc, Little Chalfont, UK). The emulsion-coated slides were stored desiccated, in a light proof box at 4°C for 2–3 weeks. Slides were then developed (D19 developer; Kodak, Rochester, NY, USA) and fixed (30% sodium thiosulphate) photographically to produce visible silver grains over the sites of hybridization. The sections were stained with haematoxylin and photographed under both light and dark field illumination using a Leica DMRB/E microscope with a 35 mm camera attachment.

Results

First trimester

Tissue sections containing first trimester villi and decidua were investigated. Most notable was the patchy flt gene expression within the trophoblast layers of the first trimester placental villi. Cross sections through some villi were heavily labelled with probe while others were lightly labelled or negative. The villus presented in Figure 1A is from a placenta at week 9 of gestation and illustrates this phenomenon within the trophoblast layers of one villus. When flt mRNA was observed in the villous trophoblast it was present in both the cytotrophoblast and the syncytiotrophoblast (Figures 1A and 1B). Apart from a small sector marked (*) the trophoblast layer of Figure 1A was intact when examined under bright field microscopy. Integrity of the trophoblast layers was also demonstrated by the staining of a serial section for cytokeratin (Figure 1C). Trophoblast on the side marked 'L' (Figure 1A) showed markedly lower levels of flt hybridization. The dense area of silver grains at the bottom of the photograph (Figure 1A) is a grazing section through the villous trophoblast as evidenced by serial sectioning which reveals the villous core in this area (Figure 1H). Similar patchy flt mRNA production in the villous trophoblast was observed in all sections from tissue up to and including week 14 of pregnancy but not later in gestation. Controls with the flt sense probe gave only diffuse background silver grains (refer to Figure 2A for an example).

Immunostaining for flt (Figure 1E) detected stronger immunoreactivity in association with the cytotrophoblast than with the syncytiotrophoblast. Immunostaining of the endothelial cells within the villi was also observed. flt positive cells were evident within the stroma of the villus and serial sections stained with CD68 suggested the localization was associated with Hofbauer cells (Figure 1F) as well as some stromal cells. Control sections with the equivalent concentration of rabbit IgG or after absorption with the flt protein resulted in no staining. On ovarian sections the flt antibody gave an expected staining pattern over only defined cell types.

In-situ hybridization with the KDR antisense probe detected message over the endothelial cells within the villi (Figure 1G). This labelling was not detected with the sense probe (Figure 1D). No KDR signal was found in association with the trophoblast.

VEGF antibody staining of the villus (Figure 1H) revealed a very similar pattern to that of the flt antibody (Figure 1E) in the trophoblast and endothelium. In the villous core, cells staining strongly for VEGF showed a similar distribution to serially sectioned and positively stained CD68 cells (Figure 1I). Other stromal cells were lightly positive for VEGF. IgG and absorption controls were negative while ovarian sections gave an expected staining pattern.

Where the villi attach to the maternal decidual tissue the flt antisense probe hybridized to the villous trophoblast and to isolated cells within the decidual tissue (Figure 2B). The distribution of these cells within the decidual tissue closely matched that of extravillous trophoblast (EVT) as determined by cytokeratin staining of serial sections (Figure 2C). There was no similarity to the distribution pattern for macrophages as determined by CD68 staining (not shown). The control sense probe gave only background silver grains (Figure 2A).

Decidual tissue samples from first trimester pregnancies showed varied tissue morphology reflecting the heterogeneity intrinsic to this material. VEGF and flt were most evident in decidual samples containing attached villi and thus at the interface between fetal and maternal tissue. In-situ hybridization with the antisense probe for KDR did not detect message for this gene within the decidual tissue. The flt antisense probe hybridized to cells within the decidua. Positive cells showed the same distribution pattern as the cytokeratin positive cells, in serial sections, and have therefore been identified as EVT (Figure 3A–D). Glandular epithelium within the decidual tissue also stained for cytokeratin and thus the distinction between glandular epithelium and intravascular trophoblast was based on morphology. The gland in Figure 3C may be slightly

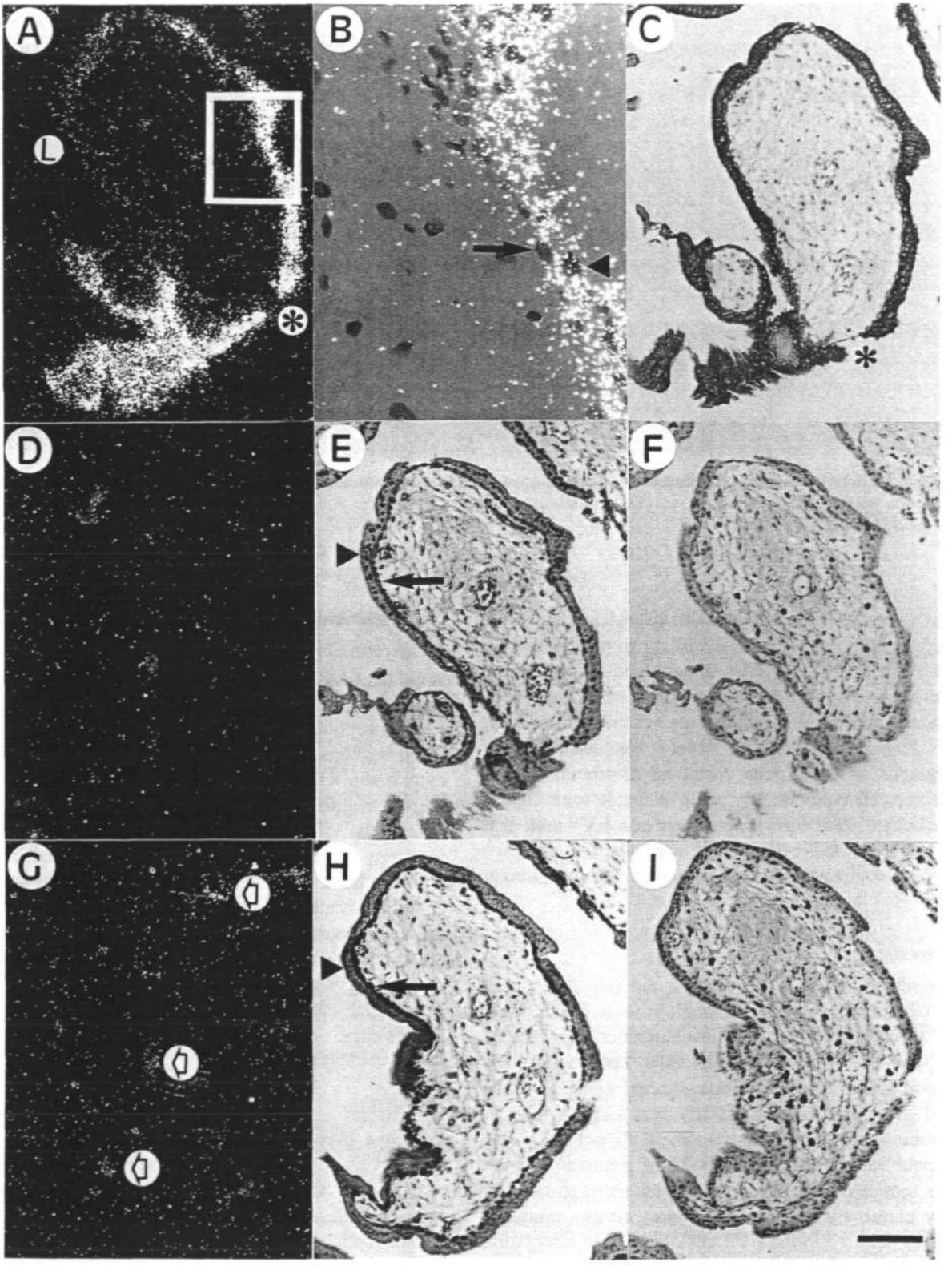

Figure 1. Sections of the same placental villus on week 9 of gestation. (**A**) In-situ hybridization with the flt antisense probe. A tear in the trophoblast is marked with an asterisk also indicated in 1C. L = low levels of flt expression in this area of the trophoblast. (**B**) Higher magnification of box section in 1A. Silver gains are visible over both cytotrophoblast (arrow) and syncytiotrophoblast (arrowhead). (**C**) Immunolocalization of cytokeratin in a serial section to 1A indicating the presence of trophoblast around the villus. Asterisk marks the same tear in the trophoblast as seen in 1A. (**D**) Sense probe for kinase insert domain-containing receptor (KDR) showing only scattered silver grains. The flt sense probe was very similar. (**E**) flt antibody staining with darker localization over the cytotrophoblast (arrow) than the syncytiotrophoblast (arrowhead). (**F**) CD68 immunostaining to localize macrophages in a serial section to 1E. (**G**) In-situ hybridization with KDR antisense probe. Open arrows indicate some of the blood vessels where the probe hybridizes. (**H**) Vascular endothelial growth factor (VEGF) immunolocalization. As in 1E the staining is darker in the cytotrophoblast (arrow) than the syncytiotrophoblast (arrowhead). (**I**) CD68 immunolocalization in a serial section to 1H. Scale bar: **A, C–I** = 100 mm; **B** = 25 mm.

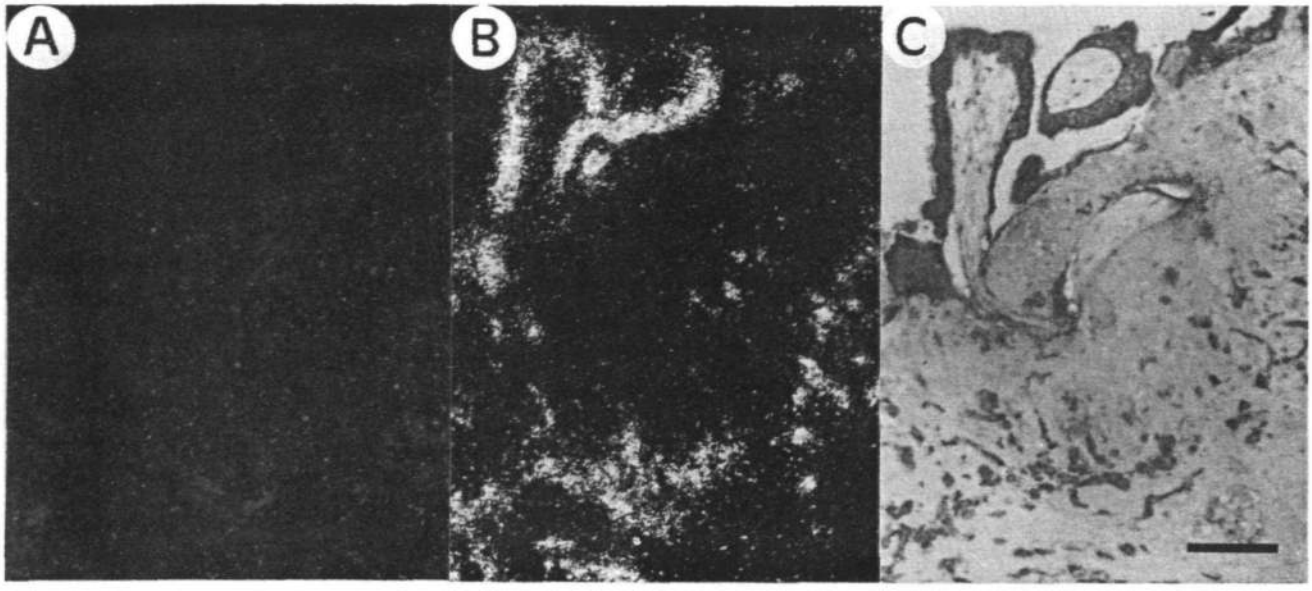

Figure 2. Serial sections of a villus attaching to the decidua on week 8 of gestation. (**A**) In-situ hybridization with the sense probe for flt. (**B**) Hybridization with the flt antisense probe. (**C**) Immunohistochemistry for cytokeratin which localizes both villus and extravillous trophoblast. Scale bar = 100 mm.

positive for flt as determined by in-situ hybridization. Blood vessels present within the decidua, without trophoblast, were negative for flt mRNA. Within some blood vessels large cytokeratin-positive ovoid cells were observed which produced flt mRNA and these were identified as intravascular trophoblast. Immunohistochemistry for the flt receptor suggested that the EVT and glands of the decidua contained flt protein (Figure 3E). The other cell types weakly positive for flt were CD45-positive leukocytes (not shown) and large non-EVT cells that are likely to be decidual cells. VEGF antibody staining gave a very similar localization pattern to that of the flt antibody (Figure 3F).

Second trimester

In-situ hybridization for KDR mRNA did not detect any significant labelling in the villi, although in some places small clusters of probe hinted that low level production might be associated with the villus endothelial cells. Endothelial cells of large blood vessels, in the decidua adjacent to the placenta, were found to label for KDR mRNA (not shown). The flt receptor probe hybridized strongly to the EVT but not to the villous trophoblast during weeks 18–22 of gestation (Figure 4A). Serial sections were stained for cytokeratin to confirm the identity of the EVT cells (not shown). flt-like immunoreactivity was detected in the EVT cells but not in association with other cell types within the maternal tissue (Figure 4C). Some very light flt staining was evident in the villous trophoblast and cells of the villous core (Figure 4C). VEGF antibody staining was found in association with the EVT and the villous trophoblast (Figure 4B). As was found in the first trimester placenta the staining in the cytotrophoblast was darker than in the syncytiotrophoblast. There was no evidence, at this stage of gestation, of strong VEGF or flt antibody staining in association with Hofbauer cells.

Third trimester

In the term placentae examined, no hybridization was detected using the KDR antisense probe. Strong expression of flt mRNA was found in EVT cells and weak, variable expression in villous trophoblast (Figure 4D). Trophoblast was identified in serial sections, to the flt in-situ hybridization slides, using a cytokeratin antibody (Figure 4E and H). When examined at higher magnification the probe was not evenly distributed around the villous trophoblast (Figure 4G). flt-like immunoreactivity indicated that the protein was present in association with the EVT cells, the trophoblast and more weakly on the endothelial cells (Figure 4F). The VEGF antibody stained both the syncytiotrophoblast and remaining cytotrophoblast, with the latter appearing to stain slightly more strongly. Other areas of staining were the EVT, the Hofbauer cells (not shown), endothelial cells and a diffuse staining present within the villous core.

Discussion

We have previously shown the presence of VEGF and its receptor flt at various stages of human placentation (Sharkey *et al.*, 1993; Charnock-Jones *et al.*, 1994). In this paper we more fully describe the localization of VEGF and its receptors within both villi and decidua, during various stages of gestation.

KDR receptor was found to be expressed most strongly on the endothelial cells of the first trimester placenta. This coincides with the period when the villi have been newly vascularized (Benirschke and Kaufmann, 1995). Mature endothelial cells fail to form when the KDR gene is inactivated (Shalaby *et al.*, 1995) and thus the expression of KDR in the developing blood vessels of the placenta may be associated with the differentiation and growth of the newly-formed

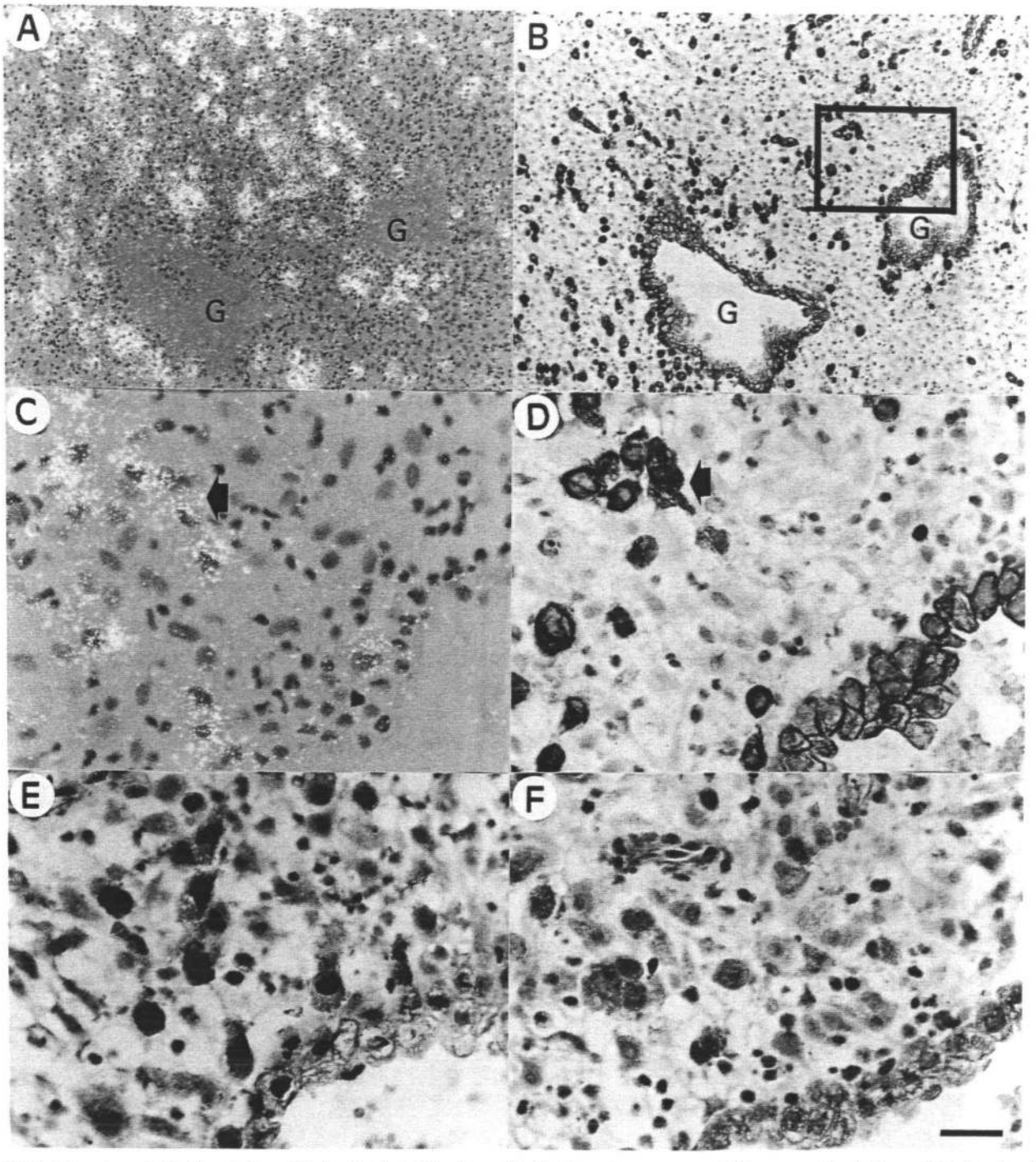

Figure 3. Decidua at week 8 of gestation. (**A**) In-situ hybridization with the flt antisense probe. Silver granules indicate high levels of flt expression. (**B**) Cytokeratin immunostaining for extravillous trophoblast (EVT) and glandular epithelium (G) in a serial section to A. (**C** and **D**) Higher magnification of boxed region indicated by B. Solid arrows indicate that the flt expressing cells in C are also the cytokeratin positive EVT of D. (**E**) flt immunolocalization within a closely-related area to the box in B. (**F**) Vascular endothelial growth factor (VEGF) immunolocalization with the glandular epithelium and decidua. Scale bar: **A–B** = 100 mm; **D–F** = 25 mm.

endothelial cells. Grimwood *et al.* (1995) have also shown that primary cultures of first trimester endothelial cells proliferate when administered VEGF. Later in pregnancy there was the suggestion of very low levels of KDR hybridization as indicated by the localization of small clusters of silver grains in association with placental endothelial cells. In light of the fact that receptors tend to have low turnover rates and thus low levels of mRNA it is probable that there is KDR on the endothelial cells later in pregnancy.

A consistent finding was that, up to week 14 of gestation the amount of flt mRNA contained in villous trophoblast varied considerably, even within a single small villus. This suggests that its production is regulated in a specific manner and may play an important role in villous development. An additional factor to consider is the nature of the probe and antibody used to detect flt. The flt in-situ probe detects message for both soluble and membrane-bound flt while the flt antibody recognizes only the transmembrane form. Northern blotting of the

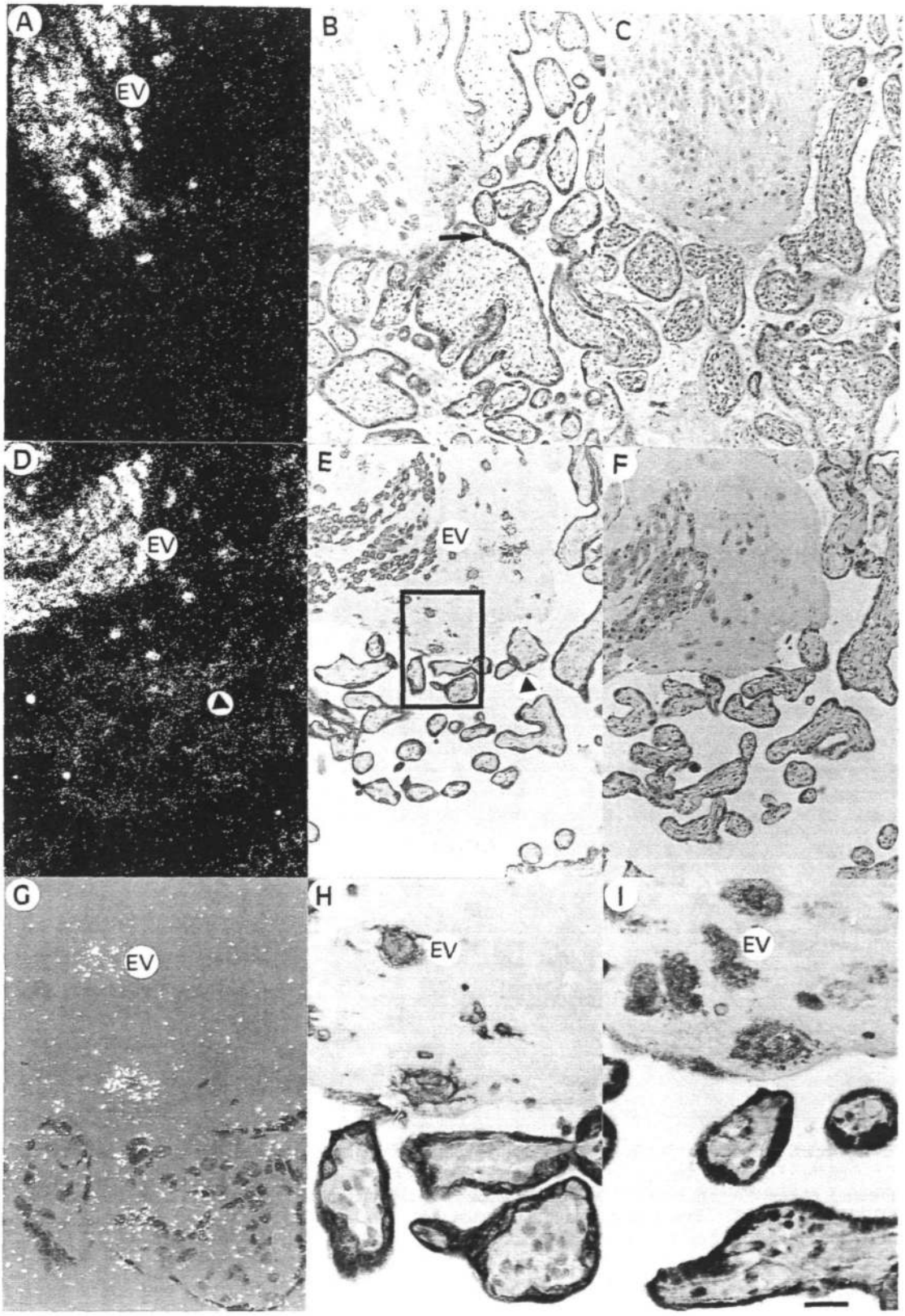

Figure 4. Placental villi and decidua at week 22 of gestation (**A–C**) and at term (**D–I**) on serial or closely related sections. (**A**) flt antisense probe hybridizing to the extravillous trophoblast (EV). (**B**) Vascular endothelial growth factor (VEGF) immunohistochemistry with strong localization on the cytotrophoblast (arrow) as well as on syncytiotrophoblast and EV. (**C**) flt antibody staining to EVT and lightly on villi. (**D** and **E**) flt antisense hybridization D and serial section labelled with cytokeratin. Strong labelling of extra villous trophoblast (EV) and light hybridization over the villous trophoblast (arrowhead) was observed. (**F**) flt immunolocalization. (**G**) In-situ hybridization with the flt antisense probe, at higher magnification. G–I are taken from the same, or related areas, to the box in E. Hybridization was seen in association with the extravillous trophoblast (EV) and lightly over the villous trophoblast. (**H**) Cytokeratin staining in a serial section to G. (**I**) VEGF immunolocalization two sections away from H. Strong staining in extravillous trophoblast (EV), villous trophoblast and lightly on the endothelial cells and cells of the villous core. Scale bar: **A–F** = 100 mm; **G–I** = 25 mm.

placental tissue has already shown that both forms are present in the placenta and that, at least at term, the soluble form predominates (Barleon *et al.*, 1994). It is possible that the villous trophoblast produces both forms and that the areas where high expression is observed correspond with cells that are making an abundance of soluble flt (Figure 1A). Those areas producing soluble flt would not be recognized by the flt antibody which was found to evenly stain the villous trophoblast (Figure 1E). Examination of flt mRNA across gestation also revealed the novel finding that in the villous trophoblast expression is down-regulated during mid-gestation and then up-regulated at term.

The presence of membrane-bound flt, by immunohistochemistry, in the endothelial cells and macrophages of the stromal core where no flt mRNA expression was observed was unexpected. This same phenomenon was observed in the decidua (Figure 3E) and to a lesser extent later in pregnancy. It must be noted that the in-situ hybridization slides were exposed for an appropriate amount of time, so as not to grossly over expose the intense areas of hybridization, and it is therefore possible that lower flt mRNA expression may have been below this detection level. Also, the turnover times for the flt splice variant proteins are not known and a combination of long half life and low mRNA levels may make some forms of flt mRNA harder to detect by in-situ hybridization.

flt mRNA was found to be produced by EVT cells throughout pregnancy. This production was therefore not dependent on whether the cells were migrating, as would be expected early in pregnancy, or more stationary, as in later pregnancy. At least some of the flt message produced by these cells is membrane bound, as evidenced by the antibody staining (Figures 3E, 4C, 4F), but whether some is also soluble remains unconfirmed. The large amounts of both mRNA encoding the flt receptor and its ligand found in the decidua, especially during first trimester (Fig. 3), would suggest that it is functionally important. It was interesting to note that only during the first trimester did the flt antibody detect other positive cell types in the decidua. Elucidating the balance and local production of soluble flt, which inhibits the activity of VEGF, and membrane-bound flt, which mediates VEGF action, will be important in understanding its functional role during placentation.

It is apparent that the VEGF protein co-localizes with the flt protein during the first trimester. The VEGF antibody used was directed against the amino terminal end and would therefore recognize all the known splice variants of VEGF. Placental growth factor (PlGF) has significant overall identity with VEGF but is not closely related in the peptide region against which the antibody to VEGF was raised (Maglione *et al.*, 1993). PlGF is of interest as it binds flt, but with much lower affinity than VEGF. PlGF can augment VEGF-induced permeability (Miles assay) and endothelial cell mitogenic activity whereas on its own, it elicits only a small response (Park *et al.*, 1994).

Immunohistochemistry identified VEGF in association with the villous trophoblast as well as more weakly on the endothelial cells, stromal cells and macrophages and is consistent with results from later in gestation. This localization was more widespread than previous reports from first trimester where Jackson *et al.* (1994) found VEGF on just the cytotrophoblast and Cooper *et al.* (1995) found VEGF only on the Hofbauer cells. It is however more in agreement with the results found in the term placenta in this paper and in previous reports of term placenta (Jackson *et al.*, 1994; Cooper *et al.*, 1995). Production of VEGF mRNA has previously been found only very weakly over the villous trophoblast but strongly in Nitabuch's layer and in decidual glands (Sharkey *et al.*, 1993; Charnock-Jones *et al.*, 1994). It is unclear whether the levels of VEGF produced by the villi could account for the amount of protein present. Other likely sources of VEGF protein are the decidual tissue and the maternal blood. The broad localization of VEGF protein reported in this study, throughout pregnancy, are not surprising considering the multiple forms of VEGF found in placenta (Sharkey *et al.*, 1993). VEGF has been shown to be produced in both intracellular and secreted forms (Ferrara *et al.*, 1992), depending on the presence or absence of the nuclear targeting sequence (Maher *et al.*, 1989). VEGF interacts with heparin and binds KDR, membrane-bound flt and at least one soluble form of flt and thus a wide distribution of protein is not unexpected (Terman *et al.*, 1992, 1994; Kendall and Thomas, 1993).

With flt now recognized as the trophoblast-associated receptor for VEGF it will be of interest to examine whether the signal transduction pathway is different in trophoblast cells and endothelial cells and what functional significance this might have.

The results presented here reveal that production and expression of this ligand and its receptors show considerable temporal and spatial changes throughout pregnancy. To better understand these changes it will be necessary to closely examine the distribution and functional significance of varying levels of soluble and membrane bound flt and the cellular consequences of flt activation within trophoblast cells.

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