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Expression of the vascular endothelial growth factor receptor, KDR, in human placenta

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ABSTRACT

Vascular endothelial growth factor (VEGF) is a heparin-binding growth factor known to act directly on vascular endothelial cells by promoting cell proliferation and permeability. To date, 3 structurally related cell surface receptors for VEGF, Flt-1, Flt-4 and KDR, have been identified and shown to be human type III receptor tyrosine kinases. The establishment of a vascular network is crucial to the development of the placenta and occurs through both angiogenesis and vasculogenesis. The signals controlling these processes are unclear. Immunohistochemical and in situ hybridisation techniques have localised VEGF in the trophoblast layers and VEGF binding to placental vascular endothelial cells and haemangioblasts has been shown, suggesting a role for VEGF and its receptors in development of the vascular network. In this study we have used specific antibodies to localise KDR and endothelial cells in 1st and 3rd trimester human placenta. The staining showed a colocalisation of KDR with endothelial cells and haemangioblasts. No staining of trophoblast cells was observed, but strong staining of the endothelial cells was seen in the villous stroma adjacent to areas of trophoblast proliferation.

Key words: Vasculogenesis; haemangioblasts; angiogenesis.

INTRODUCTION

In the growing placenta the establishment of an extensive vascular network occurs through 2 processes: vasculogenesis, which is the in situ formation of primordial vessels from haemangioblasts (blood islands); and angiogenesis, the growth of new blood vessels by sprouting from pre-existing vessels (Jakeman et al. 1993). The latter is a complex multistep process, involving endothelial cell proliferation, migration and extracellular matrix remodelling. Many factors have been reported to promote angiogenesis but only a few are known to act specifically on endothelial cells. Peptide growth factors, in particular the heparin binding growth factors (Risau, 1991), have received significant attention as regulators of angiogenesis. Acidic and basic fibroblast growth factor (a and b FGF) are well studied angiogenic factors (Klagsbrun, 1989). Even though 4 distinct receptors for the different members of the FGF family have been characterised, none of these are expressed in endothelial cells of blood vessels in vivo (Millauer et al. 1993).

Like FGF, vascular endothelial growth factor (VEGF) is also a heparin binding growth factor and has structural homology with platelet derived growth factor (PDGF) and placenta growth factor (PlGF) (Terman et al. 1992). It has been shown to promote proliferation and permeability and to be a chemoattractant of vascular endothelial cells in vitro, but is not mitogenic for other cell types including vascular smooth muscle cells and corneal endothelial cells (Terman et al. 1992; Koch et al. 1994); indeed VEGF and PlGF are the only growth factors known to act directly on endothelial cells (Ferrara & Henzel, 1989; Maglione et al. 1991). Interestingly, VEGF has also been shown to induce fetal bovine osteoblast differentiation (Midy & Plouet, 1994).

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VEGF exists in 4 forms (121, 165, 189 and 206 amino acid residues), probably as a result of alternative splicing (Klagsbrun & Soher, 1993). VEGF binds to high affinity specific receptors on vascular endothelial cells. Three structurally related human type III receptor tyrosine kinases have been cloned, flt-1, KDR and flt 4 (De Vries et al. 1992), all of which appear to be receptors for VEGF. Flt 1 and KDR have been shown to be biologically active as VEGF receptors (Klagsbrun & Soher, 1993). The KDR gene has homology with flk-1, a mouse transmembrane tyrosine kinase gene (Quinn et al. 1993) and the TKrC gene in the rat (Sarzani et al. 1992).

KDR was identified from a human endothelial cell cDNA library by the polymerase chain reaction using degenerate primers homologues to the kinase domain of known type III receptor tyrosine kinase (RTK) (Terman et al. 1992). Northern blot analysis demonstrated expression of mRNA KDR in vascular endothelial cells, although expression is variable and is lower than flt (Barleon et al. 1994). Also, while KDR expression is limited to endothelial cells, flt is expressed in nonendothelial cells including a human choriocarcinoma cell line and monocytes (Shibuya et al. 1990; Barleon et al. 1994). This is the first report of immunohistochemical staining using polyclonal antibodies to KDR in human placenta.

MATERIALS AND METHODS

Tissue preparation

First trimester human placentas (n = 5) were taken from women undergoing termination of pregnancy between 6 and 8 wk of gestation. Term placentas (n = 4) were from caesarean sections in women with uncomplicated pregnancies (38–40 wk). Placentas stored on ice were immediately transported to the laboratory. Tissue was cut on arrival and either snap frozen in liquid nitrogen and stored at −80 °C until sectioned, or was fixed immediately in cold 4% paraformaldehyde in phosphate buffered saline (PBS) or in cold 10% formalin in PBS for 24 h. Tissues were rinsed in PBS and stored in 70% ethanol prior to processing for routine paraffin embedding. Tissue sections (5 μm) were mounted on slides.

Immunohistochemistry

Polyclonal antibody to KDR was raised in rabbits as described previously (Harlow & Lane, 1988; Dougher-Vermazen et al. 1994). Briefly, a portion of the cytosolic domain of KDR was expressed in bacteria and purified as described elsewhere (Dougher-Vermazen et al. 1994). The protein was coupled to Keyhole limpet haemacyanin using glutaraldehyde (Harlow & Lane, 1988). Polyclonal antibodies were obtained from the serum of immunised rabbits using a commercial vendor (Berkeley Antibody Company; Richmond, California). The antibody was used at 1:200 dilution in PBS. Panendothelial monoclonal antibody (against CD31) was used at 1:1000 dilution in PBS (Wang et al. 1993).

Paraffin tissue sections, after deparaffinisation in xylene and rehydration, were incubated in 3% hydrogen peroxide in methanol for 20 min in order to block endogenous peroxidase. Following washes in 0.1% bovine serum albumin (BSA) (Dakopatts, Denmark) in PBS, nonspecific binding was blocked by incubation in 1.5% BSA in PBS for 20 min. The washed sections were incubated with primary antibodies (anti-KDR, anti-CD31) for 1 h, washed and incubated with secondary antibodies, horseradish peroxidase (HRP) conjugated swine antirabbit IgG at 1:100 dilution (for KDR) and HRP conjugated rat antimouse IgG (for CD31) at 1:40 dilution for 1 h. After thorough rinsing in PBS, all sections were incubated in chromogen (0.05% diaminobenzidine, 0.01% hydrogen peroxide in PBS) for 2–5 min to localise bound peroxidase. The sections were counterstained with haematoxylin for 3–5 min, rinsed in tap water for 10–15 min and mounted.

Frozen tissue sections were fixed in acetone and incubated with 1.5% BSA in PBS for 20 min to reduce nonspecific binding. The staining procedure thereafter was as above. For controls, tissue sections were treated similarly except that the primary antibody was replaced by 0.1% BSA in PBS.

RESULTS

Staining was consistently observed with both anti-CD31 and anti-KDR antibodies on sections of placental villous tissue from both the 1st and 3rd trimesters of gestation, but the intensity of staining for KDR was stronger in 1st trimester than in 3rd trimester placenta.

Anti-CD31 stained all endothelial cells in frozen sections of 1st trimester placenta. Individual cells (Fig. 1 *A*), which we presume include haemangioblasts, the endothelial cell precursors which aggregate to form capillaries during the 1st trimester (Demir et al. 1989), were strongly stained and mainly located at the edges of the villous stroma, neighbouring the cytotrophoblast cells of the trophoblast layer. Early

fetoplacental vessels were stained and were located towards the central part of villous stroma. Their size varied from only a few endothelial cells to greater numbers connected to each other by intercellular projections.

Immunohistochemical localisation of KDR in 1st trimester placenta paraffin tissue sections showed a strong presence of this VEGF receptor in the cytoplasm of endothelial cells and areas surrounding them (Fig. 1*B*). Staining was seen in apparently isolated endothelial cell as well as in the cells of blood vessels. The nuclei of the endothelial cells were not stained. In contrast to CD31 staining, where endothelial cells were clearly and distinctively stained, KDR staining was observed also in the extracellular matrix especially in mesenchymal villi where there was syncytial sprout formation and/or formation of future extravillous cytotrophoblast (Fig. 1*C*). No staining was seen in the negative controls (Fig. 1*D*).

It is interesting that individual haemangioblastic or endothelial cells were in close contact with the basal trophoblastic surface (Fig. 1*A*, *B*). Thus intimate contact between capillaries and the trophoblast layer seems to be established in newly formed villi.

In full term placenta, staining of individual endothelial cells and blood vessels revealed a very high degree of vascularisation of all types of villi in the placental tree (Fig. 2). Immunoreactivity with KDR antibodies in term placenta paraffin embedded tissue sections was as expected, all endothelial cells and the area surrounding them were stained, albeit less intensely than in the 1st trimester placenta (not shown).

DISCUSSION

This study describes the presence of KDR immunoreactivity in human placenta, demonstrating that the VEGF receptor is found on endothelial cells in vivo. KDR is also present in the villous stroma adjacent to where formation (sprouting) of cytotrophoblastic cell columns appears to be occurring. Staining with anti-CD31 antibodies confirmed that cells stained with KDR antibodies are endothelial cells (Wang et al. 1993). Due to the nature of antibodies, we were unable to perform immunostaining on serial sections (KDR antibodies gave best staining with paraformaldehyde fixed tissue, while the anti-CD31 antibody only recognises the antigen in frozen tissue); however, it is clear that it is the same cell type which is staining.

This is the first reported use of an anti-KDR antibody to localise KDR in the human placenta. VEGF is a cytokine that potently increases microvascular permeability and selectively promotes endothelial cell division and migration (Dvorak et al. 1991; Koch et al. 1994); it was therefore expected that VEGF binding sites would be found on the endothelial cells. In the term placenta, the apparent diminution of staining may reflect the reduction in endothelial cell division compared with the 1st trimester placenta, and the low vascular permeability of term placental microvasculature (Eaton et al. 1993). Demir et al. (1989) and Jones & Fox (1991) have reported that endothelial cells are located in the outer surface of the villous stroma, just beneath the trophoblast layer. Location of endothelial cells or haemangioblasts so close to the cytotrophoblast cell layer implies their close relationship. The possibility of a contribution by the cytotrophoblast cell layer to the endothelial cell population has been raised (Demir et al. 1989; Jones & Fox, 1991); however, it is now believed that the haemangioblast cells differentiate from fetal mesenchymal cells and then develop into endothelial cells. When blood island induction has occurred, the first event that follows is the formation of a primary capillary plexus from growing and fusing blood islands. The inducing signal for the development of the primary capillary plexus from blood islands is unknown (Lelkes & Unsworth, 1992).

The present study found no evidence of KDR expression in the trophoblast populations. Charnock-Jones et al. (1994) reported the presence of VEGF mRNA in cells in the mesenchyme of villi in 1st trimester placentas, and the presence of the flt receptor mRNA in trophoblast cells. This is in contrast to the work of others. Jackson et al. (1994) showed the presence of VEGF by immunohistochemistry in cytotrophoblast during the 1st trimester, and in the syncytiotrophoblast during the rest of pregnancy. Similarly, Sharkey et al. (1993) used in situ hybridisation to localise the VEGF mRNA in the villous trophoblast in 1st trimester placenta and the extravillous trophoblast at term, as well as in macrophages in the villi. Shweiki et al. (1993) and Jakeman et al. (1993), demonstrated VEGF binding to placental endothelial cells and haemangioblasts respectively. Millauer et al. (1993) localised flk-1 mRNA to proliferating endothelial cells of vascular sprouts and suggested that this receptor plays a crucial role in the development of the vascular system. Finally, Barleon et al. (1994) showed the presence of KDR in placenta-derived endothelial cells in vitro. Our results are in agreement with these latter results and similarly suggest that the trophoblast interaction with the

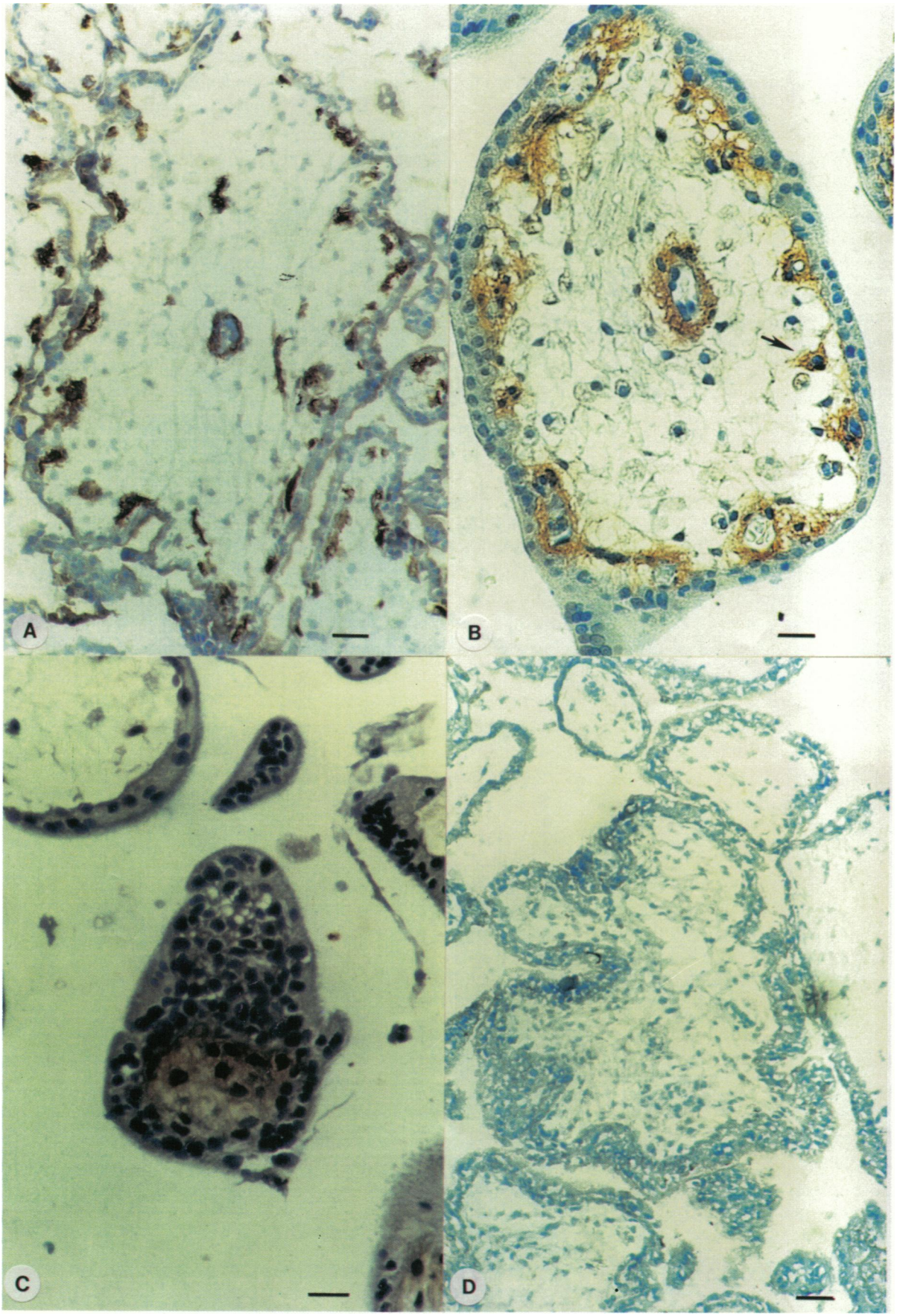

Fig. 1. First trimester human placenta stained using the immunoperoxidase procedure. (*A*) Cryostat section stained using the panendothelial monoclonal antibody, CD31. Bar, 50 μm. (*B*) Formalin-fixed paraffin embedded section stained using polyclonal anti-KDR antibody. Staining of endothelial cells in blood vessels and apparently isolated endothelial cells (arrow) can be seen. Bar, 20 μm. (*C*) As (*B*). Anti-KDR antibody also decorated the ECM of mesenchymal villi. Bar, 20 μm. (*D*) Control. Bar, 50 μm.

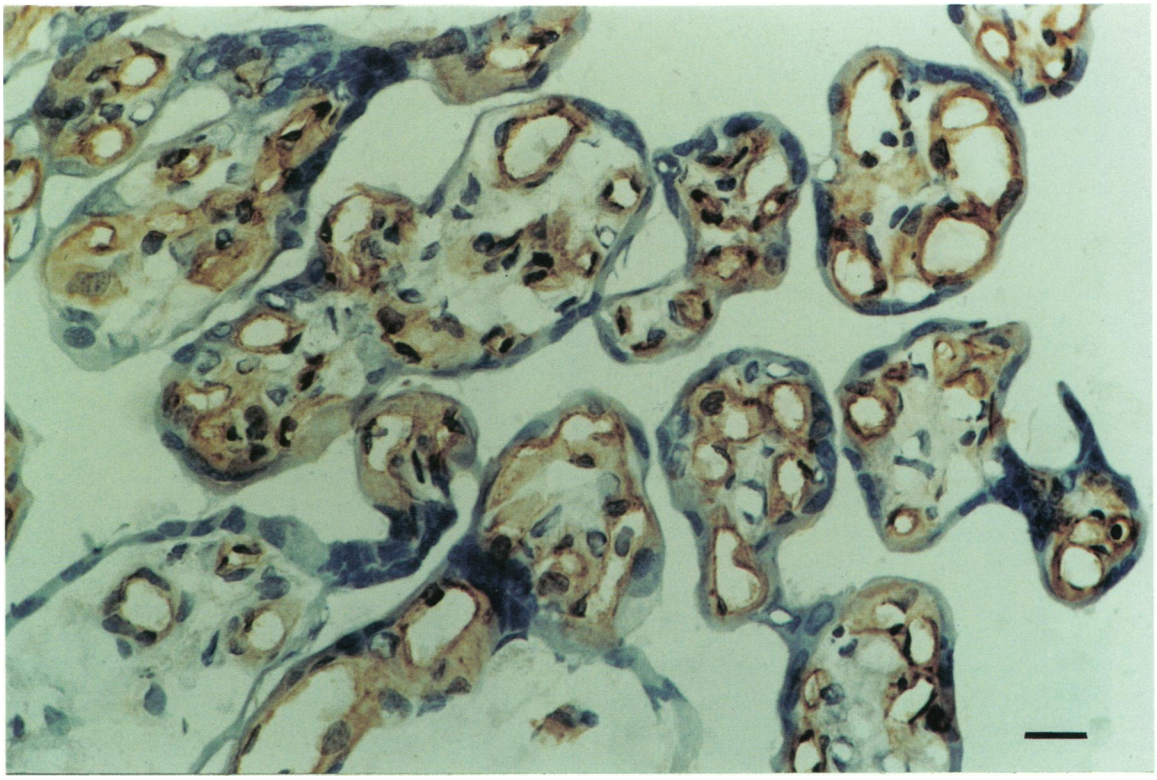

Fig. 2. Cryostat section of 3rd trimester placenta, stained using panendothelial monoclonal antibody, CD31, by the immunoperoxidase method. Bar, 20 μm.

haemangioblasts or endothelial cells may be mediated via the production of VEGF by the trophoblasts and its uptake by the developing endothelial cells. Apart from being mitogenic, VEGF may also act as a chemoattractant for the endothelial cells (Koch et al. 1994) so that the blood vessels form in close proximity to the trophoblasts, thereby maximising the maternofetal exchange of nutrients and waste products. It should also be borne in mind that, in addition to its role in angiogenesis, VEGF may have some other quite distinct function: recently it has been shown that the cytokine can induce differentiation of fetal bovine osteoblasts (Midy & Plouet, 1994).

In addition to the influence of genetic factors in the control of angiogenic and vasculogenic processes, there are environmental influences, for example the heterotypic interactions with parenchymal cells in the surrounding tissue (Graham & Lala, 1992).

Shweiki et al. (1993) reported that VEGF mRNA was found in cells surrounding the vasculature in tissues which acquire a new capillary network and it was also implied that the angiogenic process within the reproductive system (theca layers, lutein cells, endometrial stroma and the maternal decidua) is coordinated by gonadotrophins and/or by locally produced steroids and that its expression was in a cycle dependent manner (Ravindranath et al. 1992; Shweiki et al. 1993). It has also been reported that sex steroids are able to influence production of bFGF, another heparin binding angiogenic growth factor found in placenta and other reproductive tissues (Presta, 1988; Gonzalez et al. 1990; Ferriani et al. 1993). Our unpublished data (Castellucci & Vuckovic) using anti-bFGF antibodies have shown that bFGF has a similar localisation pattern to VEGF receptor in mesenchymal villi. Indeed, Pepper et al. (1992) demonstrated that VEGF and bFGF acted synergistically in inducing an angiogenic response in an in vitro angiogenesis model which assesses extracellular matrix invasion and capillary-like tube formation by endothelial cells. However, the exact function of the growth factors in the developing placenta has still to be elucidated.

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