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# Vascular Endothelial Growth Factor Receptor Localization and Activation in Human Trophoblast and Choriocarcinoma Cells[1]

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## ABSTRACT

Vascular endothelial growth factor (VEGF; also known as vascular permeability factor) is a secreted angiogenic growth factor. It is highly specific for endothelial cells, and its receptor, the *fms*-like tyrosine kinase (flt), has been localized only to endothelial cells in vivo. Here we describe the expression of mRNA encoding flt in human trophoblast as revealed by in situ hybridization. This mRNA is highly expressed in the cytotrophoblast shell and columns and also highly expressed by the extravillous trophoblast (EVT) in the maternal decidua both in the first trimester and at term. The trophoblast-like choriocarcinoma cell line BeWo also expresses this receptor and the related receptor, kinase domain-containing receptor (KDR), which is also a receptor for VEGF. Treatment of the cell line BeWo with $VEGF_{165}$ stimulated $^3$H-thymidine incorporation and tyrosine phosphorylation of MAP (mitogen-activated protein) kinase in a time- and dose-dependent fashion. This study is the first demonstration of the presence of flt on non-endothelial cells in vivo and suggests a role for VEGF in the growth and differentiation of cytotrophoblast at implantation.

## INTRODUCTION

Invasion of the endometrium by migrating trophoblast cells is essential for successful implantation and placental development. As the trophoblast is penetrating, the maternal arteries continue to elongate until well into the first trimester. Extensive angiogenesis also occurs, both within the fetal villi and in the maternal decidua, to establish the vascular structures involved in placental exchange. Some trophoblast cells invade and destroy the walls of the uterine arteries. This arterial transformation is crucial to successful implantation, allowing the increased blood flow required for the growing feto-placental unit throughout pregnancy [1]. Other trophoblast cells do not invade the maternal vasculature but remain scattered in the decidua, where they persist until term.

Vascular endothelial growth factor (VEGF) is a recently characterized angiogenic growth factor [2] that has been shown to induce vascular leakage in vivo when injected intradermally [3]; in vitro it is mitogenic for endothelial cells from a variety of sources. VEGF and its receptors, the *fms*-like tyrosine kinase (flt) and kinase domain-containing receptor (KDR) [4, 5], exhibit complementary expression patterns in fetal mice and adult rats [6, 7]; but to date they have been localized only on endothelial cells. This is consistent with the receptor's involvement in angiogenesis both in the developing embryo and in adult tissues where angiogenesis occurs, such as the luteinizing ovary [8]. VEGF and flt have also been implicated in angiogenesis associated with tumor growth, where VEGF is induced by hypoxia in glioblastoma cells [9]. Finally, the expression of flt in non-proliferating adult endothelium suggests that VEGF plays a role in endothelial cell maintenance and/or permeability [7].

In order to investigate the role of VEGF and its receptor in early placentation, we sought to localize the message encoding the flt receptor in the first trimester human placenta.

## MATERIALS AND METHODS

### *In Situ Hybridization*

In situ hybridization was performed on cryostat sections fixed in paraformaldehyde as described previously [10]. $^{35}$S-Labeled RNA probes were synthesized by in vitro transcription from a plasmid DNA clone containing the flt cDNA polymerase chain reaction (PCR) product shown in Figure 2A. Antisense and sense (control) cRNA probes were derived from opposite strands of the same plasmid. After autoradiography and development, the sections were stained with hemalum. Immunohistochemical staining of cytokeratin was carried out as described previously [10] on acetone-fixed cryostat sections using anti-cytokeratin antibody (Dako, Carpinteria, CA). Antibody binding was visualized with biotinylated anti-mouse antisera followed by avidin-

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biotin complex (ABC) peroxidase (Vectastain, Peterborough, UK).

*RNA Preparation and cDNA Amplification*

Total RNA was prepared from cell cultures by guanidine thiocyanate lysis and acid phenol extraction [11]. After reverse transcription (RT), a fraction of cDNA was amplified with two rounds of PCR using primers directed against a portion of the flt coding sequence that is poorly conserved between related receptors. This constrained the selection of primer sequence, so touch-down PCR in the nested step was used to enhance specificity. First-round amplification (25 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C) was performed in a volume of 50 μl and used primers GCAAGGTGTGACTTTTGTTC and AGGATTTCTTCCCCTGTGTA. First-round PCR product (1 μl) was reamplified via an OmniGene thermal cycler (Hybaid, Teddington Middlesex, UK) in a total volume of 50 μl by 5 cycles of 30 sec at 95°, 30 sec at x°C, and 30 sec at 72°C (where x is equal to 48, 47, 46, 45, and 44 in successive cycles) followed by 20 cycles of 30 sec at 95°C, 30 sec at 66°C, and 30 sec at 72°C. The primers, with the flt-specific sequences underlined, which included restriction sites used were GCGCTCGAGAGCATCACTCAG and GCGCGGCCGCAGTAAAATCCA. For amplification of a portion of the cDNA encoding KDR, the following nested primers were used: first round—AC(AG)(TC)TGACATG(CT)AC(AG)GTCTA and TTCCCA(CT)TTGCTGG CATCATA; second round—CTCGAATTCA(CT)CACATCCA(CG)TGGTA(CT)TGG and n ATCGAATTCGCCAAGCTTGTACCA(CT)GTGAG. These primers were designed to amplify both human KDR and its mouse homologue flk but not to hybridize to related receptors such as flt c-*kit* or c-*fms*. The internal primers contain an additional 9 nucleotides at their 5′ ends, including an *Eco*RI site to facilitate subsequent cloning. The first round used 25 cycles of 30 sec at 95°C, 30 sec at 58°C, and 30 sec at 72°C; the second used 20 cycles of 30 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C.

$^3$H-Thymidine Incorporation

Cells (BeWo; American Type Culture Collection, Rockville, MD) were plated at a density of 30 000 per well in Ham's F-12 medium containing 10% fetal calf serum (FCS). After 48 h, the medium was replaced with medium containing 1% FCS and $^3$H-thymidine (0.2 μCi/well; Amersham PLC, Bucks, UK), and incubation was continued for 18 h with $VEGF_{165}$ (2 ng/ml) [12], anti-VEGF neutralizing monoclonal antibody [13] (2 mg/ml), epidermal growth factor (EGF; 30 ng/ml), or VEGF and antibody together. The medium was then removed and the cells were fixed with 10% cold trichloroacetic acid (TCA) at 4°C for 15 min. After washing with ethanol/ether (3:1), the TCA-precipitated material was redissolved in 0.2 M NaOH and the radioactivity determined by liquid scintillation counting.

*Tyrosine Phosphorylation*

BeWo cells were grown to confluence in Ham's F-12 medium containing 10% FCS; before stimulation, the cells were starved overnight in serum-free medium. After stimulation with $VEGF_{165}$, the reaction was terminated by rapid aspiration of the growth medium followed by three washes with ice-cold buffer containing 50 mM HEPES, 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA, and 2 mM $Na_3VO_4$. Hot sample buffer (70°C) was added to the cells, and the cell contents were solubilized by passing the sample repeatedly through a syringe needle (G21). The samples were boiled for 5 min and then stored at −80°C until use. Samples (20 μl) were run on SDS-PAGE (10%) and electroblotted onto nitrocellulose. The blot was blocked for 3 h in Tris-buffered saline (150 mM NaCl, 50 mM Tris) containing 0.2% Tween (NaTT) and 3% BSA at room temperature; the blot was then incubated overnight in NaTT containing 0.2% BSA and 100 ng/ml of a rabbit polyclonal anti-phosphotyrosine antibody (Affiniti, Nottingham, UK). The blots were then washed repeatedly with NaTT (four times, 15 min each) and then incubated in NaTT containing 0.2% BSA and a 1:6000 dilution of anti-rabbit horseradish peroxidase (HRP)-linked immunoglobulin (Amersham) for 60 min. The blots were rinsed three times in NaTT and then washed for a further 2 h (eight times, 15 min each). The blots were then developed using ECL detection as detailed by the manufacturer (Amersham PLC, Bucks, UK).

## RESULTS

In situ hybridization using an flt antisense RNA probe labeled with $^{35}$S-UTP showed strong and specific hybridization to cells within the human placenta. Panels a and e of Figure 1 show brightfield views of first trimester and term placenta. The dark silver grains are clearly visible over the extravillous trophoblast (EVT) cells (marked E in panels a and e) and the cytotrophoblast shell around a villus (V in panel a) and at the base of a column (C in panel a). Darkfield views of these same sections are shown in panels b and f, the silver grains appearing as bright spots. In these two panels, weak hybridization can be seen over the villous cytotrophoblast. Although it is much weaker, this signal is specific, since sections hybridized with the sense control probe (labeled to the same specific activity) show very little signal (panels c and g). The hybridizing cells were identified as trophoblast by the staining of an adjacent section with anti-cytokeratin (panels d and h; first trimester and term placenta, respectively).

The results of hybridization with a probe to detect VEGF mRNA are shown in panels i and j of Figure 1; the sense negative control is shown in panel k. Cells within the maternal bed (i.e., on the maternal site of the zone of necrosis, known as Nitabuch's layer, marked N in panels a and i) contain large amounts of the mRNA encoding VEGF. We have previously shown that these cells are macrophages [15].

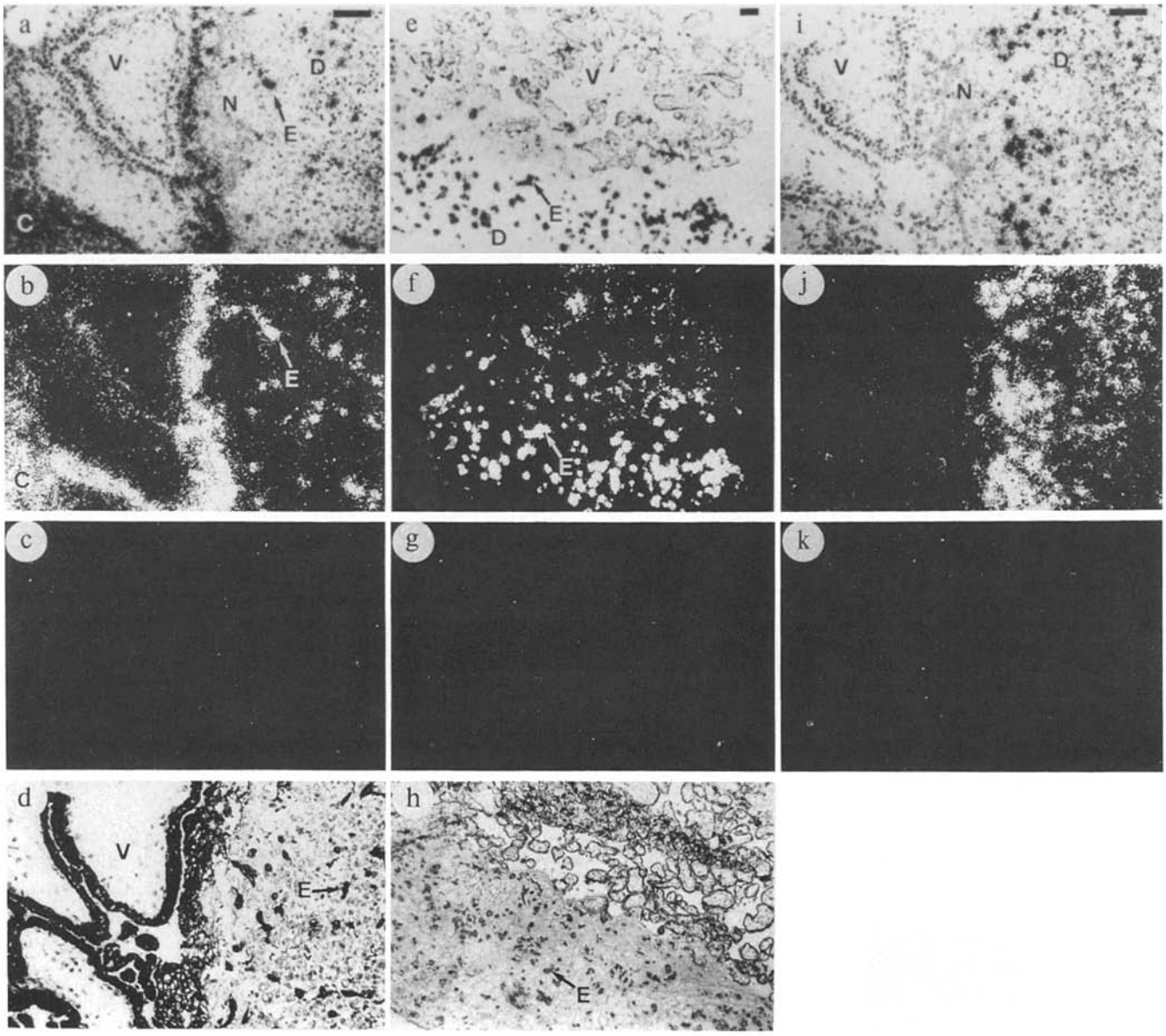


FIG. 1. Detection of human flt mRNA by in situ hybridization in human placenta. Human implantation site at 9 wk **(a-d)** and term **(e-h)**. For each site, four panels are shown: 1) a brightfield view of a section hybridized with the flt antisense probe, to illustrate tissue morphology **(a and e)**; 2) a darkfield view of the same section **(b and f)**; 3) a darkfield view of an adjacent section probed with the control sense probe **(c and g)**; 4) a serial section stained with anti-cytokeratin antibody to identify trophoblast cells. Villous trophoblast (V) is shown adjacent to maternal decidua (D), separated by Nitabuch's layer (N), a zone of necrosis that occurs at the fetal/maternal interface. The cytotrophoblast shell is seen as a cytokeratin-positive layer of cells on the fetal side of Nitabuch's layer **(panels b and d)**. The highest level of flt mRNA expression is seen in EVT cells (labeled E) in trophoblast columns and in individual trophoblast cells migrating through the maternal decidua. These cells are strongly positive for cytokeratin, as shown in **panel d. Panels i, j, and k** show sections from the same tissue block as **panels a-d**, hybridized with antisense VEGF probe **(i and j**, brightfield and darkfield, respectively) and sense VEGF probe **(k**, darkfield of section adjacent to that shown in **i and j)**. This shows the distribution of cells expressing VEGF mRNA within the maternal decidua, for comparison with cells expressing the flt receptor mRNA [15]. Scale bar represents 100 μm. A total of three implantation sites and six term placents were examined, and the results illustrated are representative of these.

Therefore the cells that produce VEGF are of maternal origin, while the cells that contain receptor and are thus able to respond are fetal.

In order to investigate the possible function of VEGF upon trophoblast cells, we tested a variety of cell lines for the presence of the mRNA encoding receptors for VEGF. The results of RT-PCR using primers specific for the two known receptors for VEGF, flt and KDR, are shown in Figure 2. The nested PCR amplification produced bands of the predicted size in each case, and their identities were confirmed by DNA sequencing. The RT-PCR analysis showed that of the choriocarcinoma lines, BeWo contained both

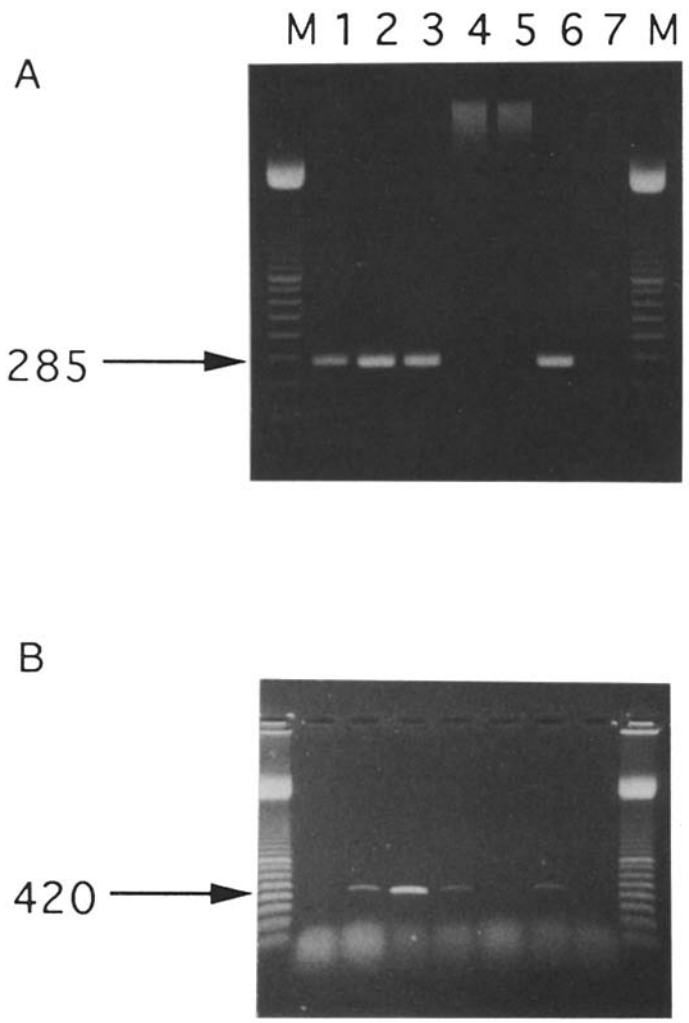


FIG. 2. A) PCR amplification of flt cDNA after RT of RNA [29] from the indicated cell lines. Lanes 1 and 2: choriocarcinoma JEG and BeWo [30 and 16, respectively]; lanes 3, 4, and 5: adenocarcinoma Ishikawa, HEC1-A, and HEC1-B [ATCC]; lane 6: human umbilical vein endothelial cells [31]; lane 7: negative control (no cDNA added). Amplification of genomic DNA (data not shown) failed to show any product indicating the probable presence of one or more introns between the primers used, confirming that the products observed here are derived from mRNA. Molecular weight markers (lane M) were the 100-bp ladder (Pharmacia Fine Chemicals, Piscataway, NJ). After digestion with *Xho* I and *Eag* I and cloning, the PCR products were sequenced and the band of 285 bp (arrowed) showed complete identity with the published flt sequence. B) Amplification by nested RT-PCR of cDNA from the same cell types as described for (A) but using KDR-specific primers.

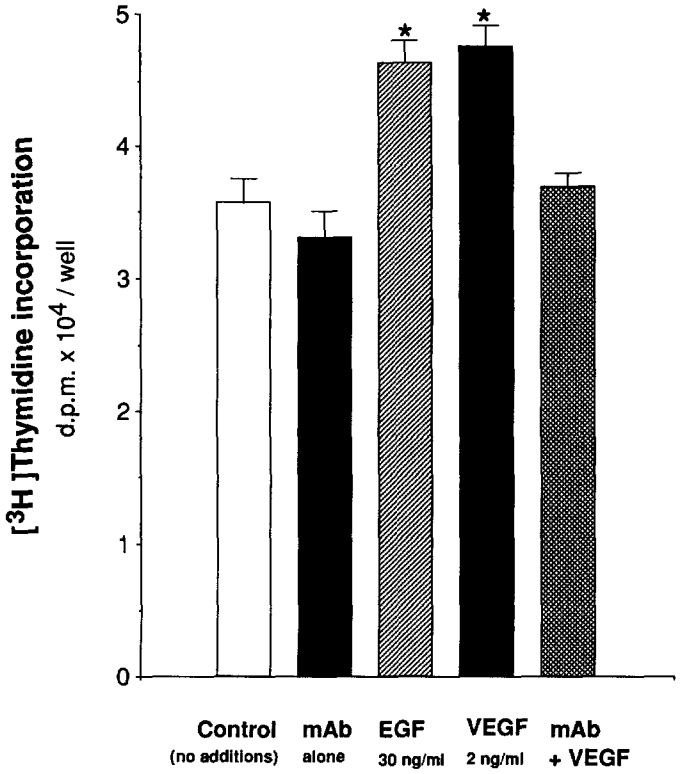


FIG. 3. Effect of recombinant $VEGF_{165}$ [12] on $^3$H-thymidine incorporation in the human choriocarcinoma cell line BeWo. Each bar represents the mean ± SEM of quintuplicate determinations from three separate experiments. Asterisk indicates a significant difference from the untreated controls, $p < 0.05$ (Student's *t*-test).

mRNAs while JEG contained only the mRNA encoding flt. Among the endometrial adenocarcinoma lines, Ishikawa contained both mRNAs, HEC 1A contained KDR but not flt, and HEC 1B contained neither. Human umbilical vein endothelial cells, as expected, contained both mRNAs.

Since VEGF stimulates cell division when applied to endothelial cells, we sought to investigate the effect of VEGF on cultured choriocarcinoma cells. Figure 3 shows that addition of recombinant human $VEGF_{165}$ to cultured BeWo resulted in a weak but significant stimulation of $^3$H-thymidine incorporation. This effect was abolished by the simultaneous addition of an excess of anti-VEGF monoclonal antibody. The magnitude of the stimulation obtained with exogenous VEGF was similar to that obtained when the same cells were treated with EGF.

The receptors for VEGF, flt and KDR, are both tyrosine kinases. Activation of cells with VEGF would be expected to result in specific time- and dose-dependant phosphorylation of tyrosine residues within specific proteins (probably including the receptor itself). BeWo cells were cultured and stimulated with VEGF (20 ng/ml) for varying lengths of time (Fig. 4a) or with varying doses for 10 min (Fig. 4b). Immunoblotting using an antibody that specifically detects phosphotyrosine showed that proteins of 42, 80, 105–120, and ~150 kDa were phosphorylated in a dose- and time-dependent manner. The clearly visible 42-kDa protein was shown via anti-MAP kinase antibodies to be the 42-kDa isoform of MAP kinase (Fig. 4c).

## DISCUSSION

We have previously identified VEGF in the endometrium [14], and in view of the importance of angiogenesis in the developing placenta we have now investigated the role of

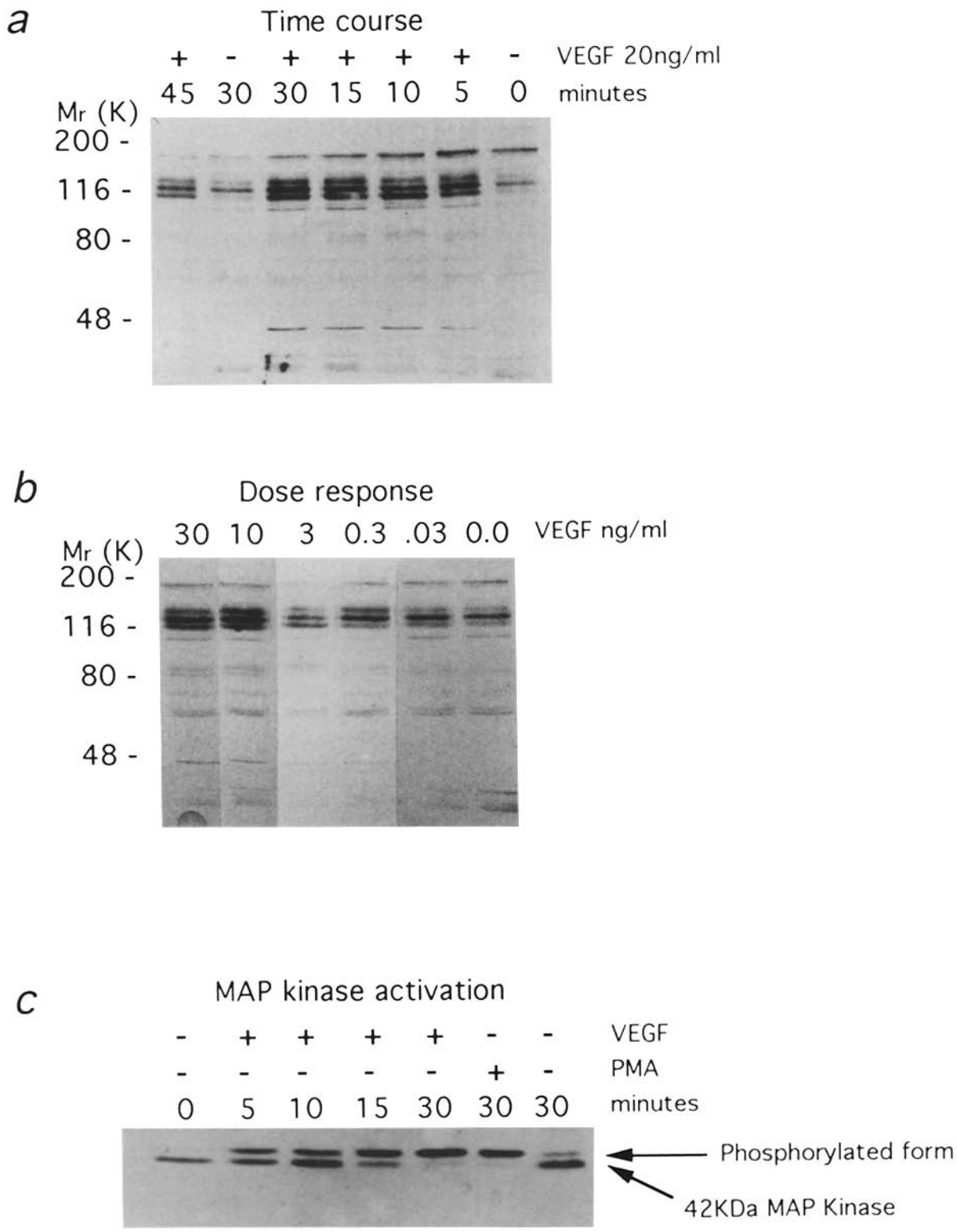


FIG. 4. VEGF-evoked protein tyrosine phosphorylation in BeWo, a human trophoblast cell line. a) Cells were stimulated with VEGF (20 ng/ml) in a time-dependent fashion. b) Cells were exposed to increasing concentration of VEGF. c) Cells were exposed to VEGF (20 ng/ml) and the lysates immunoblotted. MAP kinase (native and phosphorylated) was detected using a specific antibody directed against MAP kinase. Incubation of the cells with phorbol 12-myristate 13-acetate (PMA) was used as the positive control for activation.

VEGF and its receptor, flt, in this tissue. We previously showed that the primary sites of VEGF expression in the placenta are fetal Hofbauer cells within the villi and maternal macrophages in the maternal decidua adjacent to the site of implantation [15]. In situ localization of flt mRNA at the implantation site showed strong but specific hybridization to fetally derived EVT columns (C in Fig. 1b) and to the trophoblastic shell surrounding the embryo. Trophoblast cell proliferation occurs at the base of trophoblast columns, but cells migrating away from the base of the column cease to divide. These isolated cells then migrate toward and into the maternal decidua, which contains numerous cells synthesizing VEGF (Fig. 1j). These maternal cells are almost certainly macrophages as evidenced by their localization and morphology [15]. The EVT cells in the decidua express flt strongly in the first trimester and continue to do so at term (Fig. 1, e-h).

These data are in contrast to those reported by Millauer [16] and Jakeman [17], who showed (by in situ hybridization with an flk-1 probe or by $^{125}$I-VEGF ligand binding) that VEGF receptors are localized on hemangioblasts (blood islands) and endothelial cells in mouse and rat placenta, respectively. Our data clearly show strong hybridization with the flt riboprobe to both intermediate and terminally differentiated EVT. Thus the role of VEGF in placental development may differ between these species.

We sought to investigate the effects of VEGF using the trophoblast-like choriocarcinoma cell line BeWo [18]. These cells have some of the characteristics of EVT and express the mRNA encoding both flt and KDR (Fig. 2, A and B) [19]. Since flt expression is observed at the base of the trophoblast columns where proliferation occurs and VEGF is mitogenic, albeit for endothelial cells, we ascertained whether the addition of recombinant $VEGF_{165}$ to subconfluent cultures of BeWo cells would stimulate $^3$H-thymidine incorporation. This treatment resulted in a 30% stimulation of $^3$H-thymidine uptake, which could be specifically blocked by the addition of an anti-VEGF neutralizing monoclonal antibody [13] (Fig. 3). The degree of $^3$H-thymidine incorporation stimulated by VEGF in endothelial cells is similar to that reported by Connolly [20], and the choriocarcinoma cells are stimulated a comparable amount by EGF. Thus the relatively modest stimulation observed in these cells is probably a genuine effect. While the proliferating cells at the base of the trophoblast columns hybridize strongly to the flt antisense probe, the isolated and non-proliferating EVT cells in the decidua also are strongly positive. Thus in vivo it is possible that stimulation of cell proliferation is not the major result of flt activation in trophoblast. VEGF has also been isolated and characterized independently on the basis of its permeability-inducing action in vivo. [3]. In addition, VEGF has been shown to induce protease synthesis and release from endothelial cells [21, 22]. Therefore it is possible that these or other as yet undefined actions may be more important in the placenta.

The flt receptor is a tyrosine kinase; its activation results in auto-phosphorylation of the receptor [23] and would be expected to lead to phosphorylation of other specific proteins. Immunoblotting with anti-phosphotyrosine antisera revealed several proteins that are specifically phosphorylated in response to VEGF stimulation. The 42-kDa band has been identified as the 42-kDa isoform of MAP kinase [24]. This enzyme is believed to play a pivotal role in the regulation of growth and division [25, 26] and is phosphorylated on tyrosine as part of the growth factor-initiated tyrosine kinase cascade [27]. The phosphorylation of this protein appears to be relatively short-lived. Auto-phosphorylation of the flt receptor has been reported in HUVECs [23] and probably accounts for the band observed at 180 kDa. The other proteins that are tyrosine phosphorylated in VEGF-stimulated cells have yet to be characterized.

We have previously shown that VEGF is expressed by cells in the mesenchyme of first trimester fetal villi and in the decidua by maternal macrophages and glandular epithelium [15]. This suggests that VEGF is involved in the extensive angiogenesis within the villi and decidua during this period. However, we have now identified human trophoblast as a novel site of flt expression and shown that VEGF stimulates both $^3$H-thymidine uptake and tyrosine phosphorylation of MAP kinase in the non-endothelial cell BeWo, which is frequently used as a model for EVT. Therefore, at implantation, VEGF may also play a previously unsuspected role in the growth and migration of trophoblast. BeWo cells contain mRNA encoding both of the VEGF receptors, flt and KDR; therefore, the effects observed on $^3$H-thymidine uptake and tyrosine phosphorylation could be mediated by either or both of these receptors. However, the in situ data unequivocally demonstrate high levels of expression of flt mRNA by trophoblast in vivo.

Only a small proportion of trophoblast at the base of the columns proliferate, but these stem cells cannot be isolated by current methods. As cells progress up the column, proliferation ceases and cells differentiate into invasive trophoblast. This is accompanied by changes in cell adhesion [28]. Trophoblast cells are clearly of epithelial origin and remain cytokeratin-positive; but during implantation they contain flt mRNA, which to date has been found to be restricted to cells of endothelial lineage. These EVT then migrate through the decidua, past macrophages expressing VEGF (Fig. 1i). In view of the continued expression of flt by these invading, non-proliferating cells, we suggest that the primary role of VEGF may be in controlling the migration and differentiation of EVT rather than their proliferation. Disruption of these processes can have severe consequences for the developing fetus and mother. Preeclampsia, which affects 5–10% of first pregnancies, appears to be associated with inadequate invasion or with premature differentiation of the invading trophoblast [28]. It will be interesting to see whether the expression of VEGF

by macrophages or of flt by trophoblast is abnormal in such pregnancies.

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