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Vasodilator-stimulated phosphoprotein expression and its cytokine-mediated regulation in vasculogenesis during human placental development

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Vasculogenesis and the subsequent step, angiogenesis, are the most important stages for the continuity of placental development. Vasodilator-stimulated phosphoprotein (VASP) has a widespread role in the control of cell motility and participates in filamentous actin formation. We hypothesized that VASP participates in vasculogenesis and angiogenesis, by regulating endothelial cell migration. We therefore studied *VASP* expression in vasculogenic sites in placenta throughout pregnancy and the effect of vascular endothelial growth factor (VEGF) and interleukin (IL)-8 on the regulation of VASP expression in placental explant cultures. We found that VASP is expressed in a spatially and temporally regulated manner by various cells of the villi. In the villous stroma, the most intense immunoreactivity was observed in vasculogenic areas and in endothelial cells. In the second and third trimesters, endothelial cells demonstrated weaker immunoreactivity for VASP compared to samples from first trimester. Ultrastructural analysis of corresponding sites for VASP showed that this protein was increased in pre-endothelial cells. Areas of the strongest VEGF and IL-8 expression by villous trophoblasts corresponded to the areas of strongest VASP expression by endothelial cells, and VEGF and IL-8 showed a stimulatory effect on VASP expression in placental explants ($P < 0.05$). These results suggest that VASP may participate in vasculogenesis and endothelial sprouting during placental vasculogenesis. In addition, one of the effects of VEGF and IL-8 in angiogenesis may be to induce VASP expression in a paracrine manner.

Key words: interleukin-8/placental development/VASP/vascular endothelial growth factor/vasculogenesis

Introduction

Vasodilator-stimulated phosphoprotein (VASP), a member of Ena-VASP family, is a 46 kDa membrane-associated protein that was first described in human platelets (Halbrugge and Walter, 1989). Members of the Ena-VASP family share common amino acid sequence patterns including an EHV1 N-terminal domain that binds to proteins containing a E/DFPPPPXD/E motif and targets family members to focal adhesion (Niebuhr *et al*., 1997). This family of proteins has been studied in many systems and appears to have a universal role in the control of cell motility and intracellular actin dynamics via a linear pathway from receptor–ligand interaction (Machesky, 2000). It has been shown that VASP phosphorylation correlates well with the reversible inhibition of integrin $\alpha_{IIb}\beta_3$ (also known as fibrinogen receptor or platelet glycoprotein IIb-IIIa) (Horstrup *et al*., 1994). In an intracellular environment, VASP is associated with filamentous actin formation and is involved in intracellular signalling pathways of integrin–extracellular matrix (ECM) interaction. It is therefore suggested that this molecule takes part in cell adhesion and motility (Halbrugge *et al*., 1992).

Following blastocyst implantation, placental development necessitates vasculogenesis and establishment of vascular networks. Two processes are involved in blood vessel formation during placental development: initially vasculogenesis occurs, during which a primitive vascular network is constructed from pluripotent mesenchymal progenitors, and at a later stage, angiogenesis begins where pre-existing vasculature sends out capillary sprouts for new vessel development. Endothelial cells take part dynamically in both processes. Differentiation of multipotent mesenchymal cells, migration and proliferation of endothelial cells, as well as cell–cell connection are the most important steps for a successful vasculogenesis (Demir *et al*., 1989; Hanahan, 1997). Thereafter the vessel structure is transformed into mature vasculature through angiogenic remodelling (Demir *et al*., 1989; Suri *et al*., 1998). Fetal vascularization of human placenta is the result of local de-novo formation of capillaries, which have emerged from pluripotent mesenchymal precursor cells in the placental villi rather than sprouting of embryonic vessels into the placenta. This process starts around day 21 post-conception when the embryo is at the 4-somite stage (Knoth, 1968; Demir *et al*., 1989).

Recent studies have shown that the attachment of endothelial cells to ECM proteins via different integrin subtypes is one of the possible mechanisms for a successful vasculogenesis and placental development (Drake *et al*., 1995; Risau and Lennon, 1988). On the other hand, many paracrine factors such as vascular endothelial growth factor (VEGF) and interleukin (IL)-8 are potent angiogenic

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factors (Koch *et al*., 1992; Millauer *et al*., 1993). Presently little is known regarding the regulation of early placental vasculogenesis and angiogenesis. Since one mechanism that may explain endothelial cell migration and vascular tube formation is related to regulation of actin dynamics, we have hypothesized that VASP may take an active part in endothelial cell migration and formation of vascular networks in the human placental villous tree where vasculogenesis occurs. In the present study, we investigated the expression of VASP in haemangiogenic cells and in presumptive endothelial cells of vasculogenic areas of the placental villous core, as well as in mature vessels in diverse locations and at various stages of placental development. Furthermore, we carried out ultrastructural analysis of corresponding placental villi by electron microscopy during the early days of the pregnancy. As VEGF and IL-8 are potent angiogenic factors, immunohistochemical staining for these cytokines was performed in adjacent sections of VASP immunoreactive slides to reveal the local relationship between these proteins. Lastly, we investigated the effect of VEGF and IL-8 on VASP expression in placental explants *in vitro*.

Materials and methods

Collection of tissues

Human placental tissues from first trimester (n = 12), second trimester (n = 4) and term (n = 6) were collected from clinically normal pregnancies, which were either voluntarily terminated by dilation and curettage or delivered by Caesarean section before the initiation of labour for obstetric reasons. Informed consent was obtained from each patient before obtaining the placenta. Consent forms and protocols were approved by the Human Investigation Committees of Yale University and Akdeniz University. Pregnancy age of placental samples used in this study was calculated from the first day of the last menstrual period. Tissues were fixed in Bouin's fixative and embedded in paraffin for immunohistochemistry. Thereafter, tissues were cut at 5–7 μm thickness and mounted on gelatin-coated slides. Cryostat sections were fixed in cold methanol for 10 min. Paraffin sections were deparaffinized and rehydrated in alcohol gradient. For the detection of VASP, VEGF and IL-8, the immunohistochemistry was carried out using a standardized method based on the streptavidin–biotin technique described below.

Explant culture of human chorionic villi

Chorionic villous tissues (n = 3) from first trimester pregnancies were obtained under aseptic conditions from women undergoing voluntary termination of pregnancy (at 6–9 weeks gestation). Tissues were rinsed twice in phosphate-buffered saline (PBS), and dissected free of maternal decidua and fetal membranes. Later, tissues were minced into 2–3 mm small pieces in Hanks' balanced salt solution (HBSS; Sigma, St Louis, MO, USA). Tissue pieces were then placed in 2 ml serum-free Medium-199 (M-199) alone, or with either VEGF (2 ng/ml) or IL-8 (2 ng/ml). Explants were maintained in culture dishes at 37°C in a humidified atmosphere (5% CO_2 in air) for 24 h. Thereafter some of the tissues were snap-frozen in OCT for immunohistochemistry and the remaining tissues were used for protein extraction and Western blot analysis.

Immunohistochemistry

To detect the VASP immunoreactivity, we used a monoclonal antibody kindly supplied by Prof. U.Walter (Institute for Clinical Biochemistry, University of Wurzburg, Germany). Following the methanol fixation (for cryostat sections) or deparaffinization of sections, tissues were rinsed twice in PBS for 10 min. Endogenous peroxidase activity was quenched by 3% H_2O_2 (0.6 ml H_2O_2 and 5.4 ml methanol) for 10 min and rinsed in PBS–Tween-20 (0.05% Tween-20 in PBS, PBS-T; pH 7.3). Sections were then incubated with mouse anti-VASP (1:200 dilution), mouse anti-CD31 (1/75 dilution; Novacastra, Newcastle, UK) and mouse anti-cytokeratin 7 (1/10) monoclonal antibodies for 60 min at room temperature. Normal mouse antibody isotypes were applied as a negative control replacing primary antibodies. After several rinses in PBS-T, biotinylated goat anti-mouse IgG antibody (Biogenex, San Ramon, CA, USA) was applied for 30 min. Following several PBS-T rinses, slides were incubated with streptavidin–peroxidase complex for 30 min (Biogenex). Subsequently, the slides were rinsed several times in PBS-T, and then were incubated with 3-amino 9-ethyl-carbazole (AEC; Biogenex). Slides were mounted with a permanent-mounting medium. VEGF and IL-8 immunostaining was performed on samples collected from the first trimester using mouse anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and murine monoclonal anti-IL-8 (Bender Med Systems, Vienna, Austria).

The immunostaining intensity for VASP immunoreactivity was semi-quantitatively evaluated as positively stained target cells according to the following categories: – (no staining), 1+ (weak but detectable), 2+ (moderate or distinct), 3+ (intense).

Ultrastructural analysis

Eight samples of first trimester placental tissues used for light microscopy studies were also examined ultrastructurally. Placental tissues were from post-menstrual day 32 through to day 42.

Samples of human placental tissue were fixed by immersion in 2.5% glutaraldehyde in 0.1 mol/l cacodylate buffer (pH 7.4) at room temperature for 4 h and then post-fixed in 1% phosphate-buffered osmium tetroxide for 2 h. Specimens were dehydrated in increasing concentrations of ethanol and were embedded in an araldite epoxy resin. Semi- and ultra-thin sections were taken by ultramicrotome. Semi-thin sections were stained with Toluidine Blue. Thin sections were double-stained with uranyl acetate and lead citrate and examined with a Zeiss EM10 (Zeiss, Oberkochen, Germany).

Western blot analysis

Total proteins from explants were extracted by RIPA buffer [50 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1% Triton X-100, 0.5% sodium deoxycholic acid, 0.1% SDS, 1% protease inhibitor cocktail]. The protein concentration was determined by standard DC protein assay (Bio-Rad Laboratories, Hercules, CA, USA). A total of 10 μg protein was loaded into each lane, separated by SDS–polyacrylamide gel electrophoresis using 7.5% Tris–HCI Ready Gels (Bio-Rad Laboratories) and blotted onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). Equal loading of proteins in each lane was confirmed by staining the membrane with Ponceau 2S (Sigma). The membrane was blocked with 5% non-fat dry milk in PBS-T buffer for 1 h to inhibit nonspecific binding. The membrane was incubated for 1 h with mouse anti-VASP diluted at 1/2000. Membranes were then incubated for 1 h with peroxidase-labelled horse anti-mouse IgG (diluted at 1/10 000; Vector Laboratories, Burlingame, CA, USA). The immunoblot reactivity was developed using a TMB peroxidase substrate kit (Vector Laboratories). The VASP signal intensity was measured by laser densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

Statistical analysis

All values were expressed as mean ± SD (n = 3). Groups were compared using Kruskal–Wallis one-way analysis of variance on ranks. Statistical calculations were performed using SigmaStat for Windows, version 2.0 (Jandel Scientific Corporation, San Rafael, CA, USA).

Results

Expression of VASP, IL-8 and VEGF in placental villi throughout pregnancy

Placental villous samples were examined by immunohistochemistry to assess the VASP expression and to establish its spatial relation to VEGF and IL-8 throughout pregnancy.

Five different villous types have been described on the basis of stromal structure, cell types and vessel formation. In the order of their maturation, from the most to the least mature, these are stem villous, terminal villous, mature intermediate villous, immature intermediate villous and mesenchymal villous types (Benirschke and Kaufmann, 1995). In this study, we evaluated VASP immunoreactivity separately for each villous type (Table I). Two sections from each tissue and six to eight villi in each section were analysed for each villous type. The strongest VASP immunoreactivity was observed in placental villi of the first trimester compared with

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Table I. Vasodilator-stimulated phosphoprotein immunoreactivity in various cell types of different human placental villi throughout pregnancy

Cell type	Villous type				
	Mesenchymal	Immature	Terminal	Mature	Stem
First trimester (n = 12)					
Syncytiotrophoblast	−	−			−
Cytotrophoblast	+	+			−/+
Hofbauer cell	++/+++	++			
Haemangiogenic cell	+++	+++			
Endothelial cell	+++	++/+++			
Mesenchymal cell	++/+	++/+			
Second trimester (n = 4)					
Syncytiotrophoblast	−	−			−
Cytotrophoblast	−/+	−/+			−
Hofbauer cell	++	++			
Haemangiogenic cell	+++	+++			
Endothelial cell	++	+/++			+
Mesenchymal cell	++/+	++/+			
Third trimester (term) (n = 6)					
Syncytiotrophoblast	−	−	−	−	−
Cytotrophoblast	−/+	−	−	−	−
Hofbauer cell	++	+			
Haemangiogenic cell	+++	+++			
Endothelial cell	++	+	+	+/++	−/+/++
Mesenchymal cell	++/+	+			

placental villi of the second and third trimesters. The lowest VASP immunoreactivity was observed in cells of the stem villi, compared with those of immature intermediate and mesenchymal villi throughout pregnancy (Figure 1a–c). Independently from villous type and pregnancy age, syncytiotrophoblast and cytotrophoblast cells showed either very weak or no immunoreactivity for VASP. However, obvious differences in VASP expression were observed between stromal cells of different villous types (Figure 1a–c). The prominent finding was that VASP immunoreactivity of stromal cells decreased as placental villous tree development and maturation advanced. Thus, stromal cells of mesenchymal villi showed a stronger immunoreactivity than that of other villi. This stromal immunoreactivity was stronger in regions of villi where tendril-like villous sprouts occurred (Figure 1a).

The most relevant finding, however, was observed in endothelial cells of the vascular areas of villi. In the vasculogenic areas of the villi, the highest immunoreactivity for VASP was found in angiogenic cell cords and presumptive endothelial cells. VASP also appeared to be strongly immunoreactive in endothelial cells of newly formed primitive vascular tubes and microtubular networks of vasculature where endothelial sprouts are observed (Figure 1d,e). However, multipotential mesenchymal progenitor cells showed weaker or no staining in the same villi when compared with these cells (Figure 1e). Hofbauer cells were also strongly immunopositive for VASP. Interestingly, the endothelial cell layer and media layer of the mature vessel wall expressed weak to moderate immunopositivity for VASP in stem villi and terminal villi (Figure 1a–e). Serial immunostaining for CD31, VASP and cytokeratin revealed that there was a similar pattern of immunoreactivity for CD31 and VASP in villous stroma when compared to cytokeratin immunoreactivity in serial sections (Figure 1f–h). Moreover, CD31 immunoreactivity demonstrated clearly that presumptive endothelial and well-differentiated endothelial cells were present throughout the trophoblastic shell in villous stroma (Figures 1f and 4a insert).

IL-8 was expressed dominantly by syncytiotrophoblasts and cytotrophoblasts, whereas stromal cells showed weak or no staining (Figure 2a). However, IL-8 immunostaining intensity was spatially and temporally dependent on the villous type. The trophoblastic shell of mesenchymal and immature intermediate villi showed the strongest immunoreactivity for IL-8, while the same cell types of the stem villi only weakly expressed this protein (Figure 2a). On the other hand, both the stromal and cytotrophoblast cells were immunoreactive for VEGF. The strongest immunoreactivity for this protein was localized mainly in cytotrophoblasts of mesenchymal villi and in villous sprout areas emerging from different villi (Figure 2b). While VEGF immunostaining decreased in cytotrophoblasts as villi matured, stromal cells demonstrated increasing immunoreactivity during villous development (Figure 2b). Although cells that showed high VEGF and IL-8 immunoreactivity did not exhibit a co-localization for VASP, a relationship was observed between their expressions: the strongest VASP expression areas in stromal cells corresponded to the strongest VEGF and IL-8 expression by cytotrophoblasts in the same type of villi (Figures 1a, d, e and 2a, b).

Ultrastructural findings

During the early stages of villous formation, single or groups of haemangiogenic cells derived from undifferentiated mesenchymal cells were noticed in the villous core (Figure 3a). The haemangiogenic cells are likely to oppose each other and may form small cell clusters or cord-like cell masses, known as haemangioblastic cell cords. These were observed near the cytotrophoblastic layer of the villi (Figure 3b). Membranes of the neighbouring angioblastic cells were closely attached to each other via some cytoplasmic hills or protrusions. Among the haemangioblastic cell groups, a dilation of intercellular space surrounded by cytoplasmic hills was observed. These intercellular spaces may develop the primitive lumen of the developing vessel structure (Figure 3b). Cells with similar localization to neighbouring villous trophoblasts observed in electron microscopy showed the strongest immunoreactivity for VASP in the light microscopy (Figure 3a,b inserts). Primary capillary lumen was expanded with focal dilation of the angioblastic intercellular clefts depicted in different areas. The primitive capillary lumen appeared at the extracellular compartment, which was surrounded by presumptive endothelium (Figure 3c). Primary capillaries including haematopoietic stem cells were observed, but a capillary basal lamina could not be

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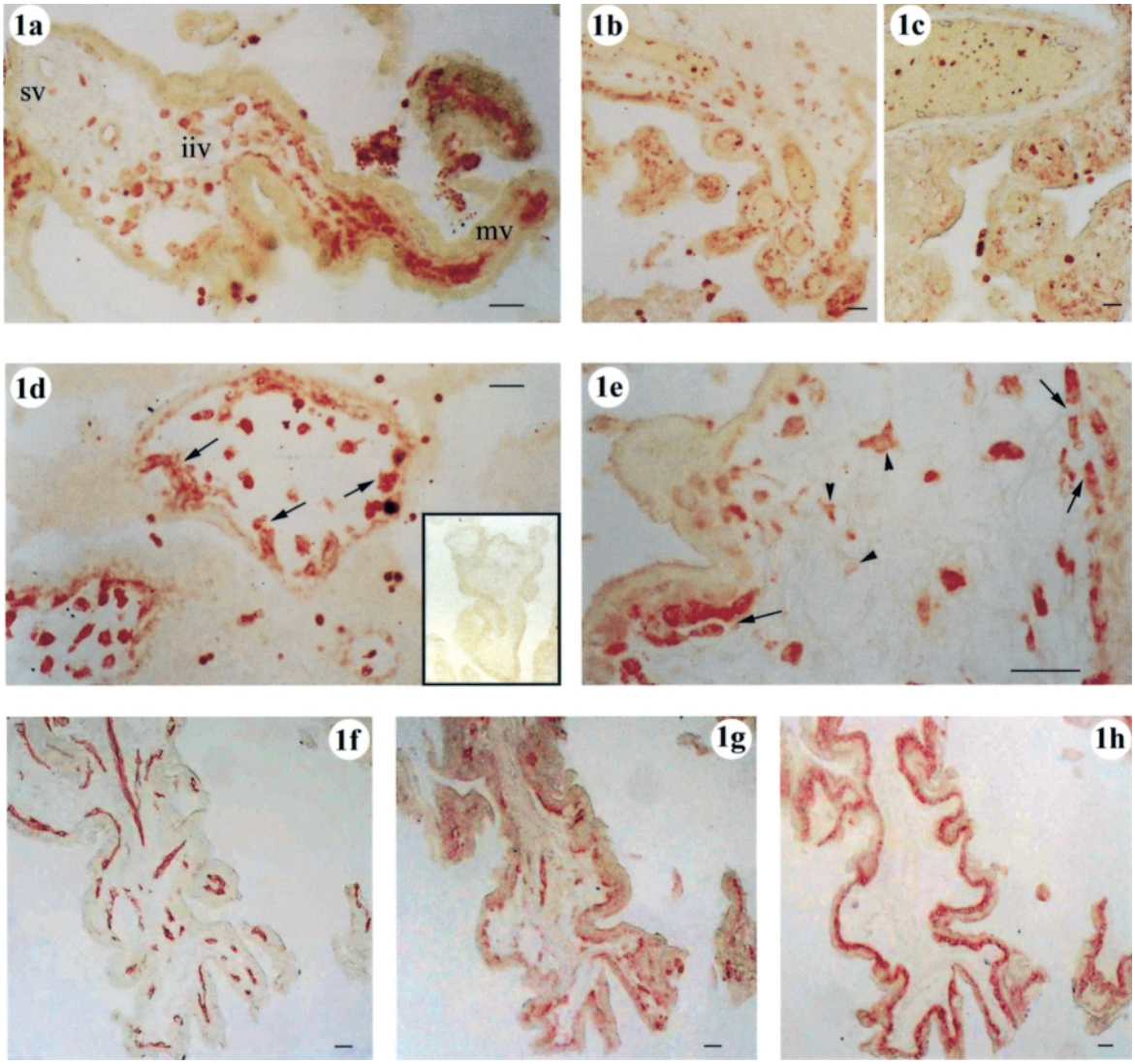


Figure 1 (**a**–**e**). Representative light micrographs of human placenta immunolabelled for vasodilator-stimulated phosphoprotein (VASP). In the villous stroma of the first trimester samples (**a**), VASP immunoreactivity was stronger than in that of second (**b**) and third trimester samples (**c**). The strongest immunoreactivity was seen in mesenchymal (mv) and immature intermediate villi (iiv) in the first trimester samples and decreasing staining was seen from the mesenchymal villous (mv) toward the stem villous (sv) (**a**). Precursor vascular cells and endothelial cells (arrows) of the newly formed vascular network where endothelial sprouts occurred were strongly immunopositive (**d**, **e**), and indifferent mesenchymal progenitor cells (arrowheads) were weak for VASP (**e**). Immunostaining of placental villous for CD31 (**f**), VASP (**g**) and cytokeratin 7 (**h**) in serial sections was observed. (**d** insert) Negative control, where primary antibody was replaced with its isotype. Scale bars = 10 μm.

detected in earlier periods of the villous development (Figure 3d). Endothelial cells with poorly developed organelles were surrounded by a varying number of precursor cells that possibly will differentiate to pericytes. In immature intermediate villi, many Hofbauer cells were found in close relation to angioblastic cell cords and primitive vascular tube formation (Figure 3c). In these areas, presumptive and migrating endothelial cells expressed VASP strongly (Figure 3c and d inserts), but in the well-developed vessels, endothelial cells demonstrated weak to moderate staining for VASP (Figure 3d insert).

Regulation of VASP by VEGF and IL-8 in angiogenic sites

Both VEGF and IL-8 are known potent angiogenic factors. Since we have observed a spatial correlation in the in-vivo expression of VEGF, IL-8 and VASP during the first trimester, we studied the effect of VEGF and IL-8 on VASP expression in explant cultures carried out with first trimester placental villi. Explants were treated with VEGF (2 ng/ml) and IL-8 (2 ng/ml) for 24 h. Both VEGF- and IL-8-treated explants showed a stimulatory effect on VASP expression in placental villi compared to control explants (Figure 4 a–c). VASP immunoreactivity was expressed strongly, especially in Hofbauer cells, and in angiogenic cell cords and presumptive endothelial cells localized close to villous cytotrophoblasts. Although it seems that the basal poles of cytotrophoblasts were also expressing VASP intensely, CD31 immunohistochemistry showed that these cells were endothelial cells lying beneath the trophoblastic shell (Figures 1f–g and 4a insert). Furthermore, Western blot analysis of explant cultures supported the immunohistochemical observations. VEGF and IL-8-treated samples showed a significant increase in VASP protein compared with the untreated control groups ($P < 0.05$; Figure 5). On the other hand, there was no significant difference in VASP phosphorylation level when VEGF- and IL-8-treated groups were compared with untreated (control) group (Figure 5).

Discussion

As a membrane-associated protein, VASP is widely distributed in the cytoplasm of migrating cells, especially at the leading edge of

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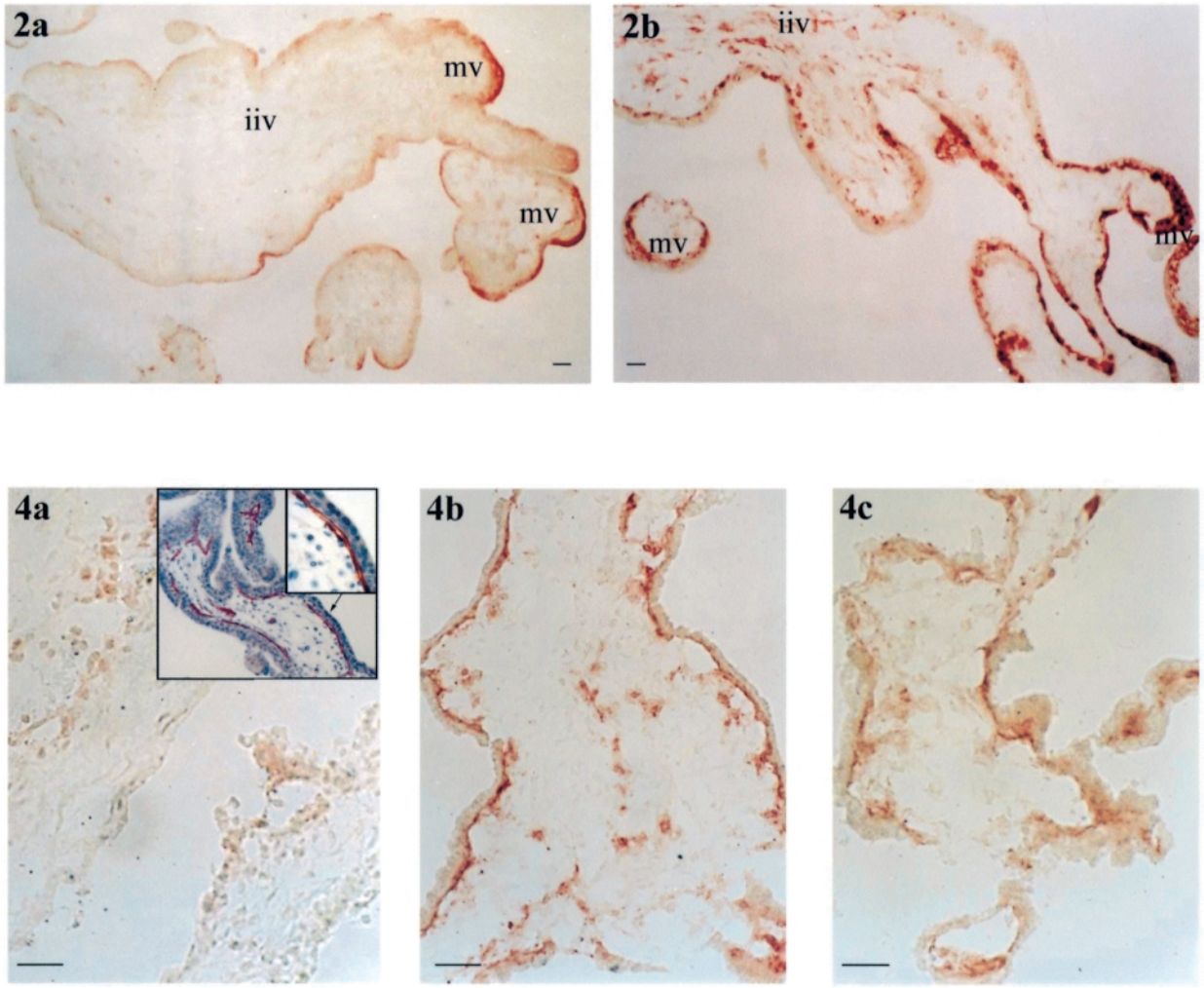


Figure 2. Representative micrographs of the human placenta immunolabelled for interleukin (IL)-8 (**a**) and vascular endothelial growth factor (VEGF) (**b**). In the first trimester chorionic villi, villous trophoblasts showed the strongest IL-8 immunoreactivity compared to other villous cells (**a**). Strong VEGF immunoreactivity was seen in both villous trophoblasts and stromal cells including Hofbauer and mesencymal cells (**b**). Interestingly, while the VEGF immunostaining was increasing in the stromal cells from the mesencymal villous (mv) to immature intermediate villous (iiv), cytotrophoblasts showed an opposite staining intensity gradient compared with stromal cells of the same villous types. Scale bars = 10 μm.

Figure 4. Representative light micrographs of vasodilator-stimulated phosphoprotein (VASP) immunoreactivity in first trimester explant cultures treated with interleukin (IL)-8 or vascular endothelial growth factor (VEGF) (**a**–**c**). Some explants were incubated with serum-free M-199 medium alone (control) (**a**), or with medium containing with IL-8 (2 ng/ml) (**b**) or VEGF (2 ng/ml) for 24 h (**c**). Explants treated with IL-8 or VEGF showed stronger immunoreactivity for VASP than did the controls. CD31 immunoreactivity revealed that the strong immunoreactivity for VASP is likely to belong to endothelial cells rather than to the basal pole of cytotrophoblasts (**a** insert). Scale bars = 10 μm.

the cytoplasm. Members of the Ena-VASP family target focal adhesion sites (Niebuhr *et al*., 1997). In many systems, VASP appears to have a universal role in the control of cell motility and in the regulation of intracellular actin dynamics via a linear pathway from a receptor–ligand interaction (Machesky, 2000). VASP is associated with filamentous actin formation and is involved in the intracellular signalling pathway of the integrin–ECM interaction. It is thus suggested that VASP participates in cell adhesion and motility (Halbrugge *et al*., 1992). In this study, we have shown that VASP has a temporal and spatial expression pattern in villous stroma depending on the villous type. VASP demonstrates increased immunoreactivity by stromal cells in mesenchymal villi and in sprouting sites of villi as compared to other villous areas. This increasing VASP immunoreactivity in parallel with the villous growth axis suggests that the protein may assist the invasion of villous sprouts by extra-embryonic mesenchyme. In addition, the weak expression of VASP by cytotrophoblasts of first trimester villi could indicate the presence of VASP in tight junction sites where cytotrophoblasts bind one another. Cytotrophoblasts in the second trimester and term placental villi were consistently not immunoreactive for VASP. Syncytiotrophoblasts, cells that are devoid of cell–cell junctions with one another, were also negative for VASP throughout gestation. This suggests a role for VASP in cell–cell junctions where it may modulate the formation of filamentous actin in cell–cell binding.

We have previously shown that VASP is expressed strongly in interstitial trophoblasts and distal extravillous trophoblasts of cell columns, implying a role for VASP in implantation and trophoblast invasion by regulating trophoblastic cell motility (Kayisli *et al*., 2002). Both endometrial receptivity and placental development demands extensive angiogenesis and vasculogenesis as well as trophoblast invasion for blastocyst implantation and successful progression of pregnancy. It has been speculated (Sharkey *et al*., 1993) that insufficient vasculogenesis or formation of imperfect vascular structures may result in intrauterine growth retardation and even in abortion. Moreover, cell type and temporal expressions of VEGF and its receptors in human placenta throughout pregnancy may reveal an

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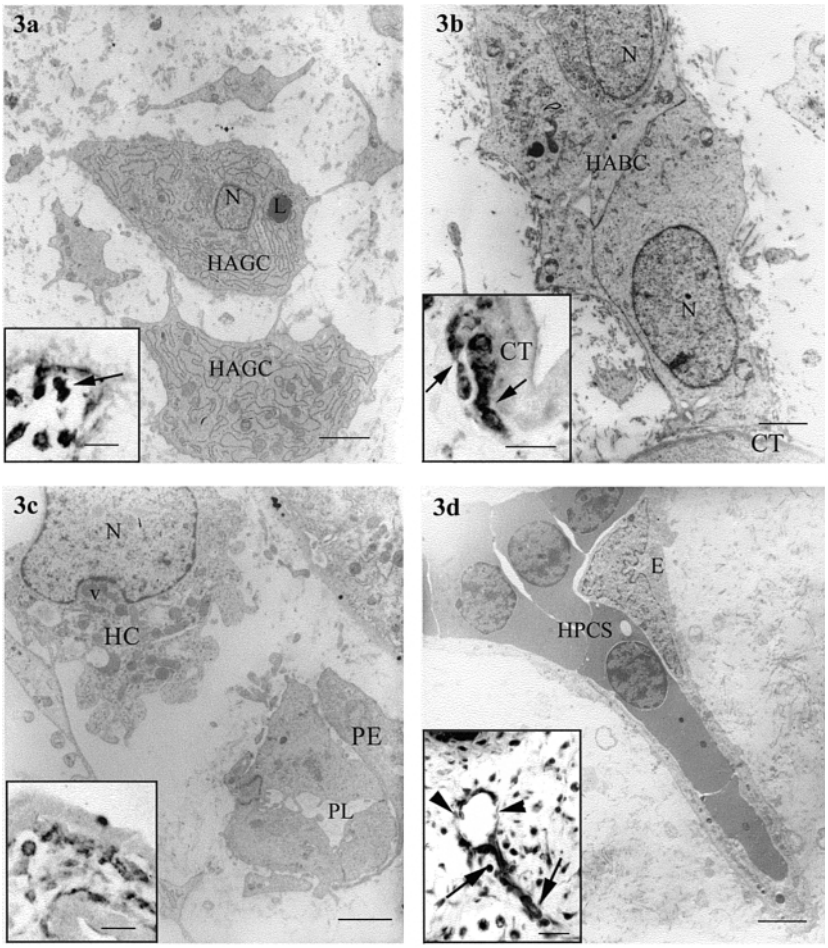


Figure 3. (**a**–**d**) Representative electron and light micrographs of vasculogenic areas in human placental villi. The haemangiogenic cells (HAGC) with the definitive cell organelles were seen in the villous core in electron microscopy and formed some cell clusters for vasculogenic centres (N = nucleus; L = lipid) (**a**). Insert pictures are not serial sections of the ultrastructural micrographs but they are from nearby areas of the same tissue. The insert shows the corresponding sites for the vasodilator-stimulated phosphoprotein (VASP) immunoreactivity (arrow) in light microscopy (insert **a**). A haemangioblastic cell cord (HABC) was seen near a cytotrophoblast cell (CT). These cells were transformed from the haemangiogenic cells (**b**) and subsequently, primitive vascular lumen (PL) lined by presumptive endothelium (PE) and a Hofbauer cell (HC) with vacuoles (v) was seen (**c**) with the corresponding sites for the VASP immunoreactivity in light microscopy (insert **b** and **c**). A mature vessel including haematopoietic cell series (HPCS), and endothelial (E) sprouts of vascular ramification was observed (**d**). Endothelial cells of the branching sites of the vessel (arrow) showed stronger immunoreactivity for vasodilator-stimulated phosphoprotein than did those of the matured sites of the vessel (arrowhead) (insert **d**). Scale bars = 3 μm; insert scale bars = 10 μm.

important role for both placental villi and maternal decidua regarding the growth, differentiation and migration of trophoblasts and endothelial cells (Clark *et al*., 1996).

Differentiation of multipotential mesenchymal cells, pre-endothelial cell proliferation and migration, together with cell–cell connections are most important subsequent steps for a successful vasculogenesis (Benirschke and Kaufmann, 1995; Hanahan, 1997). Thereafter, the angiogenic cell cords and presumptive vessel lumens lined by the vessel structure are transformed into vessel tubes and then into mature vasculature through angiogenic remodelling (Demir *et al*., 1989; Suri *et al*., 1998). The most important finding in the present study was the intensive expression of VASP by what is presumed to be endothelial cells in the vasculogenic areas and by endothelial cells of growing vessels, which send out capillary sprouts for new vessel development. This finding suggests that VASP may actively participate in vasculogenesis and vascular growth. Weak VASP expression in endothelial cells of the mature vessels supports this theory. Furthermore, Hofbauer cells, one of the sources for vasculogenic factors (Demir and Erbengi, 1984; Demir *et al*., 1989), were also found to be strongly positive for VASP. These cells are known as a type of migratory cell of the villous stroma, supporting a migratory role for VASP. In addition, the ultrastructural observations and VASP immunoreactivity are consistent, suggesting that VASP may actively play a role in haemangioblast cell migration to form presumptive capillaries. Recent studies have shown that VASP participates in endothelial cell migration. The phosphorylation of VASP results in the loss of VASP activity and in the loss of its binding partner, zyxin, from focal adhesions in endothelial cells. This phosphorylation causes up to 50% inhibition of human umbilical vein endothelial cell migration *in vitro* (Smolenski *et al*., 2000).

Many studies have shown VEGF expression and localization at both the protein and mRNA level in cells of placental villi (Sharkey *et al*., 1993; Jackson *et al*., 1994; Shiraishi *et al*., 1996). During pregnancy, VEGF has been reported to play different roles in the development of the placental and decidual vascular network (Jackson *et al*., 1994; Shiraishi *et al*., 1996), regulation of vascular permeability (Sharkey *et al*., 1993), and differentiation and migration of trophoblasts (Clark *et al*., 1996). Recently, it has been shown that cultured placental endothelial cells produce VEGF and that this autocrine secretion of VEGF induces endothelial cell proliferation *in vitro* (Bocci *et al*., 2001). IL-8-producing cells in human placental villi are cytotrophoblasts, syncytiotrophoblasts and macrophage-like cells (Shimoya *et al*., 1992; Saito *et al*., 1994). This chemokine is a potent angiogenic factor as well as chemotactic factor for leukocytes (Strieter

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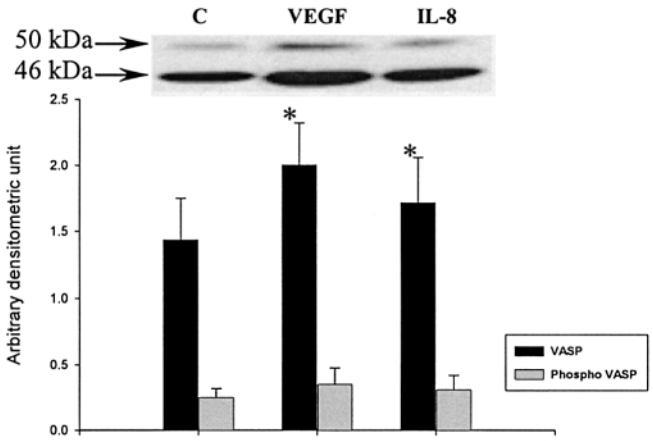


Figure 5. A representative immunoblot and densitometric quantification of the regulation of vasodilator-stimulated phosphoprotein (VASP) expression by interleukin (IL)-8 and vascular endothelial growth factor (VEGF) in first trimester placental explants. Placental explants were incubated with serum-free M-199 media alone (C: control), or with media containing 2 ng/ml IL-8 (IL-8), or with media containing 2 ng/ml VEGF for 24 h. The 46 and 50 kDa bands represent the normal and phosphorylated (phospho-VASP) forms of the VASP protein respectively. Experiments were repeated twice with consistent results using three explant cultures from three different placentae each time and all values were expressed as mean ± SD ($n = 3$). *$P < 0.05$.

et al., 1995). In line with the results of previous studies, the present study showed similar VEGF and IL-8 immunodensity in placental villi. Interestingly, VEGF and IL-8 were observed to have a spatially dependent expression. Both proteins were most strongly expressed in cytotrophoblasts of the mesenchymal villi and in its tendril-like sprouts as well as in villous sprouts that emerge from immature intermediate or stem villi. Recently, it has been shown that VEGF could be up-regulated by cytokines such as tumour necrosis factor (TNF)-α and transforming growth factor (TGF)-β1, suggesting an additional explanation for spatial VEGF expression in placental villi during pregnancy (Chung *et al*., 2000). Similarly, IL-8 has been shown to be regulated by IL-1 and by TNF-α in human placental cells (Shimoya *et al*., 1999). Although villous cytotrophoblast and stromal cells did not show a co-localization model for VASP and VEGF, or for VASP and IL-8, there was a corresponding intensity gradient between VASP–VEGF and VASP–IL-8. This correlation may be responsible for the migration route of stromal and endothelial cells in the placental villous tree. Helske *et al*. have shown that expression of VEGF receptor (VEGFR)-2 is localized almost exclusively to vascular endothelial cells in human placentae from healthy, diabetic and pre-eclamptic women (Helske *et al*., 2001). These results suggest that these cytokines may regulate VASP expression by a paracrine mechanism and may play a role in the villous development and maturation via vascularization processes. Since IL-8 is also expressed at high levels in endometrial epithelial cells (Arici *et al*., 1993, 1998; Garcia-Velasco and Arici, 1999), this may represent another paracrine mechanism for endometrial-trophoblastic interaction. Shimoya *et al*. have shown that IL-8 is expressed normally by placental explants throughout pregnancy and that the lowest expression of this protein is found in the first trimester explants when compared with those from the second and third trimester (Shimoya *et al*., 1992). In-vitro results of the present study revealed that VEGF and IL-8 had a stimulatory effect on VASP expression. This result may suggest that VEGF and IL-8 could direct migration of extra-embryonic cells in newly formed villous stroma as well as endothelial cell sprouts during vascular growth and new vessel formation by affecting the amount of VASP expressed. However, it seems that neither VEGF nor IL-8 would have a long-term effect on VASP phosphorylation. The observation was made that the presence of Hofbauer cells in angiogenic sites or vessel growth routes could reflect another paracrine mechanism for the regulation of VASP in endothelial cells since Hofbauer cells are one of the known sources of angiogenic factors such as VEGF (Cooper *et al*., 1995).

In conclusion, our results suggest that VASP is likely to take part in vasculogenesis and vascular growth by endothelial sprouts, which form new blood vessel networks. In addition, VASP may assist in villous maturation by regulating stromal cell migration into newly formed mesenchymal villous cores. Moreover, the relationships between VEGF and VASP and between IL-8 and VASP imply that one of the angiogenic and vasculogenic effects of VEGF and of IL-8 could be via stimulation of VASP expression. On the other hand, further functional and in-vitro studies are needed to better understand the function and regulation of VASP in vasculogenesis and angiogenesis in human placenta.

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