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Expression of VEGF-C and activation of its receptors VEGFR-2 and VEGFR-3 in trophoblast

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Summary. Placental villous development requires the co-ordinated action of angiogenic factors on both endothelial and trophoblast cells. Like vascular endothelial growth factor (VEGF), VEGF-C increases vascular permeability, stimulates endothelial cell proliferation and migration. In the present study, we investigated the expression of VEGF-C and its receptors VEGFR-3 and VEGFR-2 in normal and intrauterine growth-restricted (IUGR) placenta. Immunolocalisation studies showed that like VEGF and VEGFR-1, VEGF-C, VEGFR-3 and VEGFR-2 co-localised to the syncytiotrophoblast, to cells in the maternal decidua, as well as to the endothelium of the large placental blood vessels. Western blot analysis demonstrated a significant decrease in placental VEGF-C and VEGFR-3 protein expression in severe IUGR as compared to gestationally-matched third trimester pregnancies. Conditioned medium from VEGF-C producing pancreatic carcinoma (Suit-2) and endometrial epithelial (Hec-1B) cell lines caused an increased association of the phosphorylated extracellular signal regulated kinase (ERK) in VEGFR-3 immunoprecipitates from spontaneously transformed first trimester trophoblast cells. $VEGF_{121}$ caused dose-dependant phosphorylation of VEGFR-2 in trophoblast cells as well as stimulating DNA synthesis. In addition, premixing $VEGF_{165}$ with heparin sulphate proteoglycan potentiated trophoblast proliferation and the association of phospho-ERK with the VEGFR-2 receptor. $VEGF_{165}$-mediated DNA synthesis was inhibited by anti-VEGFR-2 neutralising antibody. The results demonstrate functional VEGFR-2 and VEGFR-3 receptors on trophoblast and suggest that the decreased expression of VEGF-C and VEGFR-3 may contribute to the abnormal villous development observed in IUGR placenta.

Key words: VEGF-C, VEGFR-2/(KDR), Placenta, Endothelial cells, Pregnancy, IUGR.

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Abbreviations. VEGF: vascular endothelial growth factor; VEGF-C: vascular endothelial growth factor-C; VEGFR-1: vascular endothelial growth factor receptor-1 (Flt-1); VEGFR-2: vascular endothelial growth factor receptor-2 (KDR); VEGFR-3: vascular endothelial growth factor receptor-3 (Flt-4); IUGR: intrauterine growth restriction; ED_{27}: first trimester cytotrophoblast cell line; ERK: Extracellular signal regulated kinase; HSPG: heparin sulphate proteoglycan; CM: condition medium; TTBS: Tween tris buffered saline; LD: Laser Densitometry; MAP: Mitogen activated protein

Introduction

Establishment and development of the human placenta requires the co-ordinated proliferation and differentiation of both endothelial and trophoblast cells. The feto-placental vessels formed by the processes of vasculogenesis (Risau and Lemmon, 1988), and angiogenesis (Kaufmann et al., 1985) ultimately give rise to the specialised gas exchange capillaries of the terminal villi, the functional unit of the placenta (Benirschke and Kaufmann, 1995). This is essential for establishing a circulatory system that carries up to 40% of the fetal cardiac output towards the end of pregnancy (Eik-Nes et al., 1980; Hendricks et al., 1989). The successful development and function of the feto-placental circulation is in turn dependant on the concurrent establishment of a functional trophoblast-lined maternal-placental circulation in the intervillous space (Benirschke and Kaufmann, 1995).

Impairment of these processes is thought to contribute to the condition intrauterine growth restriction (IUGR), a major cause of fetal morbidity and mortality. Elaborate ultrastructural and morphological studies of severe IUGR placenta with reduced or absent end-diastolic flow velocity (AEDFV) show an aged syncytium and an impairment of angiogenesis with terminal villi displaying straight unbranched capillaries and erthrocyte congestion (Krebs et al., 1996; Macara et al., 1996). These findings, together with the observation of higher maternal venous pO_2 in pregnancies complicated by IUGR (Pardi et al., 1992), have led to

the suggestion that in IUGR, unlike preeclampsia, there is "placental hyperoxia" (Kingdom and Kaufmann, 1997). It was subsequently proposed that increased oxygen levels surrounding the placental villi may limit angiogenesis as a result of disturbances in the expression and function of oxygen-sensitive angiogenic growth factors (Ahmed and Kilby, 1997). It should be noted, however, that changes in protein expression might also occur once the complication has developed. Nevertheless, we have shown that levels of placenta growth factor (PlGF) are elevated in IUGR placentae and that PlGF is upregulated by hyperoxia *in vitro* and that it inhibits endothelial but stimulates trophoblast proliferation (Khaliq et al., 1999). Further confirmation comes from our recent study on angiopoetin-2, another angiogenic factor that was reported to be up-regulated by hypoxia (Oh et al., 1999), showing that this factor is decreased in severe IUGR (Dunk et al., 2000). Correlation of express and functional studies strongly suggests that the abnormal morphology observed in IUGR placentae may occur as a result of an early disturbance in the relative expression levels of angiogenic growth factors during the formation of the terminal villi.

Vascular endothelial growth factor (VEGF) is an oxygen sensitive endothelial cell-specific mitogen and a potent angiogenic inducer of blood vessel formation (review, Ferrara and Davis-Smyth, 1997). In the human placenta, mRNA encoding VEGF (Sharkey et al., 1993) and VEGF receptor-1 (VEGFR-1/Flt-1) (Charnock Jones et al., 1994) and their proteins (Ahmed et al., 1995) are expressed by the macrophages of both fetal and maternal origin, endothelial cells and in villous cyto- and syncytiotrophoblast. As the terminally differentiated layer of syncytiotrophoblast (vasculosyncytial membrane) is in direct contact with maternal blood, it is assumed that they possess some endothelial functions in addition to their role as epithelial transporters. Indeed, stimulation of trophoblast VEGFR-1 is functionally coupled to endothelial nitric oxide synthase activation (Ahmed et al, 1997). In more recent years, new separate gene product members of the VEGF family have emerged including VEGF-B, VEGF-C, VEGF-D and VEGF-E (review, Ortega et al., 1998).

Like VEGF, VEGF-C is able to increase vascular permeability and to stimulate the migration of bovine capillary endothelial cells in three-dimensional collagen gels (Joukov et al., 1996, 1997). Functional studies have demonstrated that VEGF-C mediates lymphangiogenesis stimulating lymphatic endothelial cell proliferation (Fitz et al., 1997; Oh et al., 1997). VEGF binds with high affinity to both VEGFR-1 (de Vries et al., 1992) and VEGFR-2/KDR (Millauer et al., 1993) while VEGF-C binds to VEGFR-2 and VEGFR-3/Flt-4, displaying a preferential affinity for VEGFR-3 [KD = 135 pM] (Joukov et al., 1996). Proteolytic processing of VEGF-C from a 32 kDa form to a mature 23 kDa form increases the interaction with VEGFR-2 [KD = 410 pM] (Kukk et al., 1996; Joukov et al., 1997). To date the localisation of VEGFR-2 within the human placenta has been reported to be restricted to the feto-placental endothelial cells, where it is thought to promote angiogenesis (Barleon et al., 1994; Holt et al., 1997). Although VEGFR-3 is expressed on all early endothelial cells it becomes restricted to lymphatic endothelium during embryogenesis suggesting that VEGF-C mediates the formation of venous and lymphatic endothelium (Kaipainen et al., 1995; Kukk et al., 1996).

Relatively large amounts of VEGF-B and VEGF-C mRNA (Kukk et al., 1996), and two VEGFR-3 mRNA transcripts (Galland et al., 1993) have also been identified in the placenta, though their expression patterns and function remain to be determined. The aim of this study was to identify the sites of expression of VEGF-C, VEGFR-2 and VEGFR-3 in human placenta and to further quantify the gestational changes in expression levels of VEGF-C and VEGFR-3 to make a comparison between severe IUGR and gestationally matched normal placenta. As these studies revealed that trophoblast in addition to endothelial cells, expressed VEGFR-2 and VEGFR-3 we investigated functionality of these receptors in stimulating trophoblast proliferation using *in vitro* assays and a stable trophoblast cell line.

Materials and methods

Tissue collection

First trimester placental tissues (FT, 7-12 weeks gestation; n=11) were collected from surgical termination of pregnancies. Third trimester (3T, 27-36 weeks gestation; n=5) and uncomplicated term placenta (Term NL 38-42 weeks gestation; n=14) delivered by elective Caesarean section for breech presentation or a recurring indication in otherwise uncomplicated pregnancies were collected as non-labouring tissues. Labouring term placenta (Term Lab, 38-42 weeks gestation; n=12) were obtained from uncomplicated vaginal deliveries. In addition non-labouring tissues were also collected by elective Caesarean section from pregnancies complicated by IUGR (IUGR, 28-36 weeks of gestation; n=10) or preeclampsia (PE, 28-37 weeks gestation; n=10). For analysis these placenta were compared with the gestationally matched normal non labouring third trimester group. Placental tissues for IUGR were obtained from women with absent end diastolic flow velocity (Hanretty et al., 1989) and small for date babies with fetal weight < 5th centile for gestational age. Preeclampsia was diagnosed when blood pressure was at least 140/90 mmHg or greater in a previously normotensive woman after 20 week gestation, and that there was at least 300 mg/day proteinuria, platelet count < 150,000/ml and urate > 350 mmol/l (Sibai, 1991). At least two such blood pressure recordings were obtained on consecutive occasion's four hours apart. All subjects in the preeclampsia group were investigated in the puerperium to ensure that they were normotensive with no significant proteinuria at twenty

weeks post-delivery. Placental tissues were collected from a central location lying between the basal and chorionic plates and were the full thickness of the placenta. Amnion and chorion (chorionic laeve) were collected from each placenta from reflected membranes formerly covering the decidua parietalis of the uterus. A section of umbilical cord was also collected from each delivery. Cervical biopsies were also collected as a control tissue from non-pregnant patients undergoing investigative procedures. Tissues were immersed in 10% formaldehyde, embedded in paraffin wax and processed for immunocytochemistry (3 μm sections), or snap frozen in liquid nitrogen immediately after collection prior to homogenisation. Ethical committee approval for tissue collection was obtained from the South Birmingham Ethical Committee.

Antibodies

Rabbit polyclonal VEGFR-1/(Flt-1) (C-17), rabbit polyclonal VEGFR-2/(flk-1/KDR) (C-1158), rabbit polyclonal VEGFR-3/(Flt-4) (C-20) and goat polyclonal VEGF-C (C-20) antibodies were purchased from Santa Cruz Biotechnology (Autogen Bioclear, Stevenage, UK). These antibodies are raised against sequence specific peptides and demonstrate no cross reactivity between VEGFR-1, VEGFR-2 and VEGFR-3 or VEGF, VEGF-B and VEGF-D respectively. VEGFR-2 and VEGF-C western blots were run against control recombinant proteins VEGFR-2 (Flk 1158-1345), VEGF-C (sc-1881 P) (Autogen Bioclear). Anti-human mouse monoclonal cytokeratin and macrophage CD68 were purchased from Dako limited (Dako Ltd, Bucks, UK).

Immunohistochemistry

Immunohistochemistry for VEGFR-3 and VEGF-C was carried out in normal term non labouring placenta. Immunohistochemistry was carried out to localise VEGFR-2 in normal first trimester (n=5) and term non labouring (n=6), preeclamptic (n=6) and IUGR placentae (n=6). Serial 3 μm sections of formalin-fixed, paraffin-embedded placental tissues were used for immunohistochemistry as described in detail (Ahmed et al., 1995). Briefly, sections were deparaffinized and hydrated. Sections were pre-treated by microwave in 10mM citrate buffer, 0.1% EDTA (pH 6.0) for 30 minutes. Endogenous peroxidase activity was quenched by the incubation in 0.3% (v/v) of hydrogen peroxide in methanol for 10 minutes. Sections were either incubated with anti-VEGFR-2 primary antibody (1μg/ml) or anti-VEGFR-3 primary antibody (2μg/ml) or anti-VEGF-C primary antibody (4μg/ml) in 5% milk solution for 60 minutes. Amplification of the primary antibody reaction was achieved using a biotinylated goat anti-mouse/rabbit secondary antibody or a biotinylated anti-goat secondary antibody for 30 minutes followed by a complex of streptavidin and biotinylated peroxidase (DAKO Ltd, Bucks., UK) for a further 30 minutes. Finally, binding was visualised by incubating the sections with 0.5 mg/ml diaminobenzidine (Sigma Chemical Co. Ltd, Poole, Dorset, UK) and 0.01% hydrogen peroxide in PBS for 5 minutes. Between each step the sections were washed in 3x200 ml of PBS for 5 min. All incubations of antiserum were carried out at room temperature in a wet chamber. Sections were counterstained with Mayers Haematoxylin (Raymond A Lamb, London, UK), dehydrated and mounted. Control sections were performed in duplicate tissues for each antibody where the primary antibody was replaced with 10% non-immune goat serum. Additional controls for the VEGF-C and VEGFR-2 antibodies were performed where antibodies were pre-absorbed with 100-fold excess control peptides provided by Autogen Bioclear. Serial sections were also stained with anti-human mouse monoclonal cytokeratin and macrophage CD68 antibodies to identify specific cell type.

Western Blotting

Proteins were extracted from first trimester (FT, n=4), third trimester (3T, n=4), term non-labouring (NL, n=5), and labouring (Lab, n=5), intrauterine growth retarded (IUGR, n=5), and preeclamptic (PE, n=5) placenta and subjected to Western blot analysis as previously described (Ahmed et al., 1997). Briefly, extracted protein was resuspended in 2xSDS sample buffer and boiled for 5 minutes. Equal amounts of total protein (100μg) was separated on a 10% polyacrylamide gel by electrophoresis, and transferred to nitrocellulose membranes (Amersham, Bucks., UK) at room temperature, overnight. Membranes were blocked with 1% milk fat (Premier Beverages, Staffs., UK) in Tween tris buffered saline (TTBS: 10mM Tris pH7.5, 100mM NaCl, 0.1% Tween 20) for 6 hours at room temperature, washed in TTBS at room temperature for 15 minutes, and the wash repeated twice for 5 minutes. Membranes were incubated with either anti-VEGF-C antibody (0.2μg/ml) or anti-VEGFR-3 antibody (0.1μg/ml) at 4 °C, overnight. Membranes were washed and antibody reactions were detected using the ECL detection kit (Amersham, Bucks., UK), followed by detection of chemiluminescence on X-ray film. Intensity of detected bands was quantified by laser densitometry (LD) and presented as LD units.

Cell culture

A first trimester spontaneously transformed stable trophoblast cell line ED_{27}, generated by repeated passaging of trophoblast obtained from first-trimester chorionic villi by chorionic villous sampling (Diss et al., 1992), was used to assess effects of VEGF and VEGF-C. The ED_{27} cell line has been characterised and demonstrated to express the alpha and beta subunits of human chorionic gonadotrophin, placental alkaline phosphatase, and cytokeratin peptide-8 but not vimentin and factor VIII (Kniss et al., 1994). All passages used in

experiments exhibited phenotypic features consistent with cytotrophoblast.

Preparation and stimulation of cells

The first trimester ED_{27} trophoblast cell line was maintained in T25 flasks in a 1:1 mixture of DMEM/HAMS F-12 containing 15% FCS, 1% L-Glutamine and 1% antibiotic mixture (5000 IU/ml penicillin and 5000μg/ml streptomycin) at 37 °C 5% CO_2, 95% humidity. Cells were grown to 80% confluence and the medium replaced with serum free DMEM/F-12 for 24 hours. For assessment of the presence of functional VEGFR-2, cells were stimulated with increasing concentrations of $VEGF_{121}$ or $VEGF_{165}$ (Strahmann Biotech, Hannover, Germany) either alone or premixed with heparin sulphate proteoglycan (HSPG) (1μg/ml) (Sigma). For the assessment of the presence of functional VEGFR-3, cells were stimulated with conditioned medium from the human carcinoma cell lines Hec1-B and Suit-2. All experiments were performed on three independent occasions and in triplicate wells on each occasion.

Autophosphorylation of VEGFR-2 and VEGFR-3 in trophoblast cells

The first trimester trophoblast cell line ED_{27} was prepared and stimulated as described above. Controls of unstimulated cells were also carried out with serum free medium containing 0.2% BSA. Following incubations flasks were washed with ice cold PBS and protein was extracted by addition of 0.5ml immunoprecipitation buffer and precipitations carried out as described previously (Ahmed et al., 1997). Briefly lysates were precleared and protein concentrations were determined by the Biorad protein assay. Anti-VEGFR-1, anti-VEGFR-2, or anti-VEGFR-3 antibodies were added to respective experiments as indicated (1μg/ml), and incubated overnight at 4 °C on an orbital shaker. The immunocomplex was captured by addition of Protein-A 4% beaded agarose, washed three times with immunoprecipitation buffer and resuspended in 50 μl of 2x sample buffer, prior to SDS-PAGE performed on 25 μl of each sample. Proteins were transferred to a nitrocellulose filter and phosphorylated bands visualised by incubation of the filter with monoclonal anti-phosphotyrosine PY20 (0.5 μg/ml) (Affinity Research Products Limited, Exeter, UK) for one hour at room temperature. Association of the phosphorylated mitogen activated kinase (MAP) kinase ERK was assessed by western blotting of VEGFR-1, VEGFR-2 or VEGFR-3 immunoprecipitates with an anti-phospho-ERK antibody (0.025μg/ml) (New England Biolabs, Hitchin, Hertfordshire, UK). The non radioactive ECL Western Blotting System (Amersham International plc, Bucks, UK) was used to detect phosphorylated bands associated with the VEGF receptors. Results are expressed as a representative of three independent experiments.

[^{3}H] thymidine incorporation.

ED_{27} cells were seeded in 24 well plates in growth medium (DMEM/F-12 15% FCS) at a density of 30,000 cell per well and grown to 70-80% confluence. Cells were rendered quiescent by incubation for 24 h in DMEM/F-12 0.2% BSA. $VEGF_{165}$ both alone and premixed with HSPG (1 μg/ml) was added to the cells at increasing concentrations (1-50 ng/ml) and incubated for 30 h. During the last 6 h of the 30 h incubation with VEGF, cells were labelled with [methyl-^{3}H] thymidine at 0.2 μCi/ml (Amersham Int. plc). Following completion of the incubation cells were washed with PBS, fixed in 5% ice cold TCA, and washed with 100% ethanol. Cells were lysed in PBS, 0.2% BSA, 1% Triton X-100 and 1mM NH_4OH and incorporated [^{3}H]-thymidine measured by ß-scintillation counter. Cell proliferation was assessed over a 48 h time period under the same stimulatory conditions by counting cell numbers in a standardised Coulter counter (Coulter electronics, Harpenden, Herts., UK) with the lower threshold level set to 14. Results are expressed as a mean of three independent experiments (mean±S.D) each performed in triplicate determinations and were analysed by student t-test.

Results

Expression of VEGFR-3 and VEGFR-2 proteins by human placenta

To investigate specificity of the anti-VEGF receptor antibodies used total protein from first trimester and

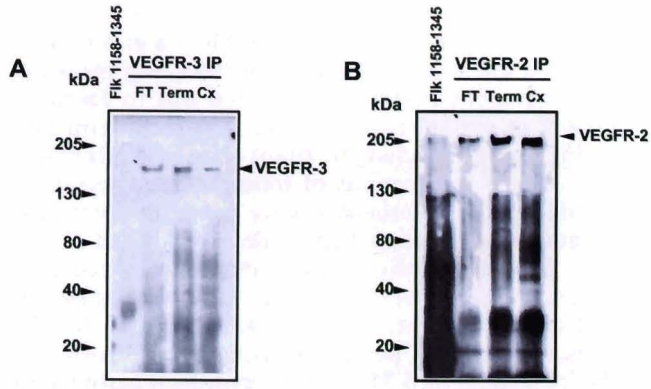


Fig. 1. Identification of the VEGFR-3 and VEGFR-2 receptors in placental extracts. Placental proteins were immunoprecipitated with either an anti-VEGFR-3 (A) or anti-VEGFR-2 antibody (1 μg/ml) followed by SDS PAGE and Western blotting with either anti-VEGFR-3 (A) or anti-VEGFR-2 (B) antibody (0.1 μg/ml) against a recombinant VEGFR-2 (Flk-1158-1345) positive control. **A.** VEGFR-3 immuno-precipitates from first trimester (FT) and Term Labouring (TL) placental, and cervix (Cx) proteins display a specific 170kDa band. No band was detected in the VEGFR-2 Flk-1158-1345 positive control. **B.** VEGFR-2 Immuno-precipitates from first trimester (FT), Term Labouring (TL) placental and cervix (Cx) proteins display a specific 210 kDa band corresponding with the recombinant Flk-1158-1345 VEGFR-2 protein.

term placenta were immunoprecipitated overnight with either an anti-VEGFR-3 or anti-VEGFR-2 antibody (1 μg/ml). Immunoprecipitates were separated by SDS-PAGE and run against a recombinant VEGFR-2 protein (Flk-1158-1345) as positive control followed by Western blotting with the anti-VEGFR-3 or anti-VEGFR-2

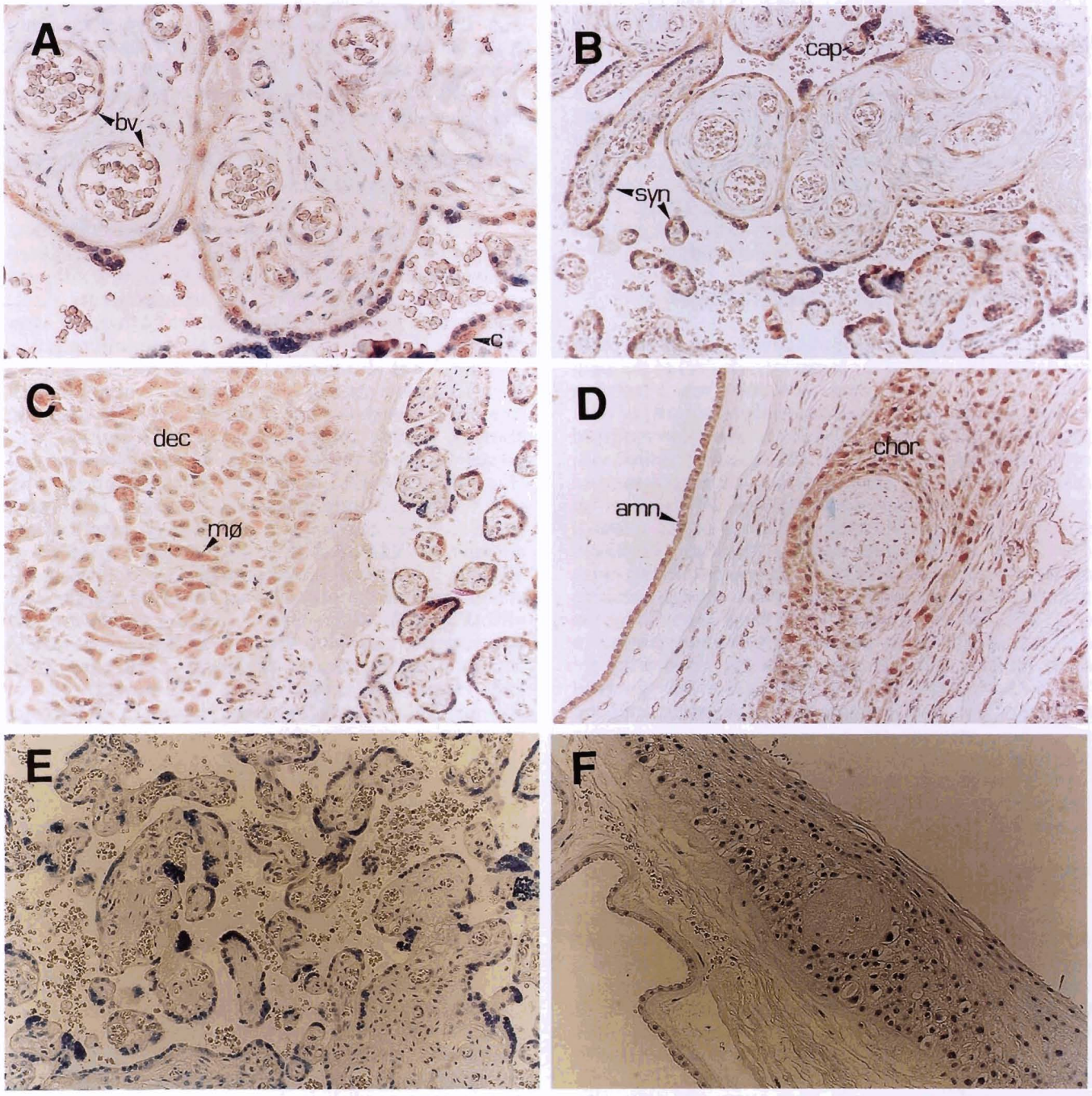


Fig. 2. Immunolocalisation of VEGF-C in normal human term placenta. **A.** Immunostaining for VEGF-C was detected in the endothelial cells of the large blood vessels in stem villi (bv) and in isolated cytotrophoblast (c). **B.** Moderate VEGF-C immunostaining of the syncytiotrophoblast of the smaller terminal villi (syn). **C.** The maternal decidua (dec) displays variable immunostaining for VEGF-C with some macrophage cells more strongly positive than others (mø). There is no staining of the extra villous trophoblast, identified by strong cytokeratin immunostaining (not shown here). **D.** The amnion (amn) and chorion (chor) show moderate staining for VEGF-C. **E.** Control sections incubated with the primary antibody preabsorbed with excess control peptide showed no immunostaining. **F.** Control sections incubated without the primary antibody showed no immunostaining. A, D, x 560; B, C, E, F, x 280

antibody (0.1 μg/ml). Placental protein VEGFR-3 immunoprecipitates demonstrate a specific 170 kDa band in both first trimester and term placenta (Fig. 1A) and is in agreement with previously published report (Galland et al., 1993). No band was detected with anti-VEGFR-3 in the VEGFR-2 fusion protein Flk-1158-1345 used as VEGFR-2 positive control, while cervix protein displayed the 170 kDa band confirming specificity of the anti-VEGFR-3 antibody (Fig. 1A) (Sisi, 1997). In contrast, VEGFR-2 immunoprecipitates display a specific band of 210 kDa in first trimester, term placenta and cervix (Fig. 1B). This molecular weight corresponds to the band detected in the VEGFR-2 positive control and agrees with previous reports (Waltenberger et al., 1994, Thieme et al., 1995). These studies confirm specificity and non-cross reactivity of the anti-receptor antibodies used in this study.

Immunolocalisation of VEGF-C in the placenta

Weak VEGF-C immunoreactivity was observed in the endothelial cells of the larger vessels (bv) of the stem villi (Fig. 2A). VEGF-C immunostaining was detected in varying intensity in the syncytiotrophoblast (syn) of term placental villi (Fig. 2B) and also in isolated cytotrophoblast (c) (Fig. 2A). The smaller terminal villi displayed stronger staining than the larger mature primary and secondary stem villi (Fig. 2B) however no staining was detected in the capillaries of the terminal villi (cap). The maternal decidua (dec) also displayed moderate to weak VEGF-C immunostaining with maternal macrophage cells (mø) more strongly positive (Fig. 2C). The extra villous trophoblast layer lining the decidua, identified by positive cytokeratin staining in serial sections (data not shown) showed no staining for VEGF-C. Moderate VEGF-C staining was also observed in the vascular chorion (chor) and the avascular amniotic epithelium (amn) in term placenta (Fig. 2D). No positive staining was observed when the antiserum was pre-absorbed by excess of VEGF-C control peptide or when the primary antibody was replaced with non-immune serum (Fig. 2E,F).

Immunolocalisation of VEGFR-3 in the placenta

VEGFR-3 protein expression in the human placenta localised to endothelial cells of arterial, venous and capillary blood vessels (bv) (Fig. 3A). Strong VEGFR-3 immunostaining was observed in the endothelial cells of the large placental vessels, and surrounding the veins of the mature stem villi (v) (Fig. 3B). The endothelial cells of the smaller placental capillaries (cap) demonstrated more moderate VEGFR-3 immunostaining (Fig. 3A). The large umbilical vessels, both artery and vein, also stained strongly for VEGFR-3 (data not shown). More interestingly, the highest level of immunostaining for VEGFR-3 was detected in the syncytiotrophoblast of the terminal villi, the functional gas exchange unit of the placenta (Fig. 3C). The trophoblastic staining demonstrated a decrease in intensity from the terminal to the mature stem villus. The maternal decidua (dec) also demonstrated strong immunostaining for VEGFR-3 though the invasive extravillous trophoblast, identified by positive cytokeratin staining in serial sections of the same tissue (data not shown), showed no staining (Fig. 3D). Term placental chorionic (chor) and amniotic (amn) epithelial membranes the also displayed strong VEGFR-3 immunostaining (Fig. 3E). No positive staining was observed when the antiserum was replaced with non-immune serum, or (Fig. 3F).

Immunolocalisation of VEGFR-2 in healthy and complicated placentae

This study demonstrated very intense VEGFR-2 immunostaining in the terminally differentiated syncytiotrophoblasts (syn) (Fig. 4) of placental villi, and in the invasive extravillous trophoblasts (evt) (cytokeratin positive, data not shown) of normal term placenta (Fig. 4B). Furthermore, immunoreactive VEGFR-2 was also detected in trophoblast cells isolated from term placenta and a first trimester trophoblast cell line ED_{27} cytospun onto a microscope slide (data not shown). Intense immunostaining for VEGFR-2 was present in the Hofbauer cells (h) of term placental villi (Fig. 4A), identified by positive immunostaining with anti CD68 antibody. Maternal decidual cells (dec) (negative for cytokeratin and CD68) also stained strongly for VEGFR-2 (Fig. 4A). Strong expression of VEGFR-2 immunoreactive protein was detected in the endothelium of the major blood vessels in mature stem villi (Fig. 4D), with a weaker staining apparent in the paravascular capillary network of the stem villi and capillary endothelium of the terminal villi (cap) (Fig. 4C). Interestingly, the pattern and intensity of the VEGFR-2 immunostaining in the placental sections from pregnancies complicated by preeclampsia (Fig. 4D) or IUGR (Fig. 4E) showed no obvious difference from normal term placenta. No positive staining was observed when the primary antibody was replaced with non-immune serum, or when the antiserum was pre-absorbed by excess of the Flk-1158-1345 control peptide (Fig. 4F).

Expression levels of VEGF-C protein in placentas from healthy and complicated pregnancies

We next investigated the expression levels of VEGF-C protein in placenta throughout gestation and in placenta complicated by IUGR and preeclampsia. Semi-quantitative western blot analysis of VEGF-C protein levels in the placenta demonstrated a major 23 kDa protein corresponding to the control VEGF-C protein in all samples (Fig. 5A), a second 32 kDa protein was detected in some samples. The size of the bands detected correspond with reported sizes for VEGF-C (Kukk et al., 1996) and provide confirmation of the specificity of the anti-VEGF-C antibody. Laser densitometric analysis of

the 23 kDa band demonstrated that levels of VEGF-C protein were highest in first trimester (white bar) and third trimester (grey bar) placenta and decreased to term in non labouring placentae (grey hatched bar) (Fig. 5B). Interestingly, at term the levels of VEGF-C demonstrated a highly significant 2 fold increase in band intensity in labouring placenta (black hatched bar) as compared to non-labouring placenta ($p<0.0001$,

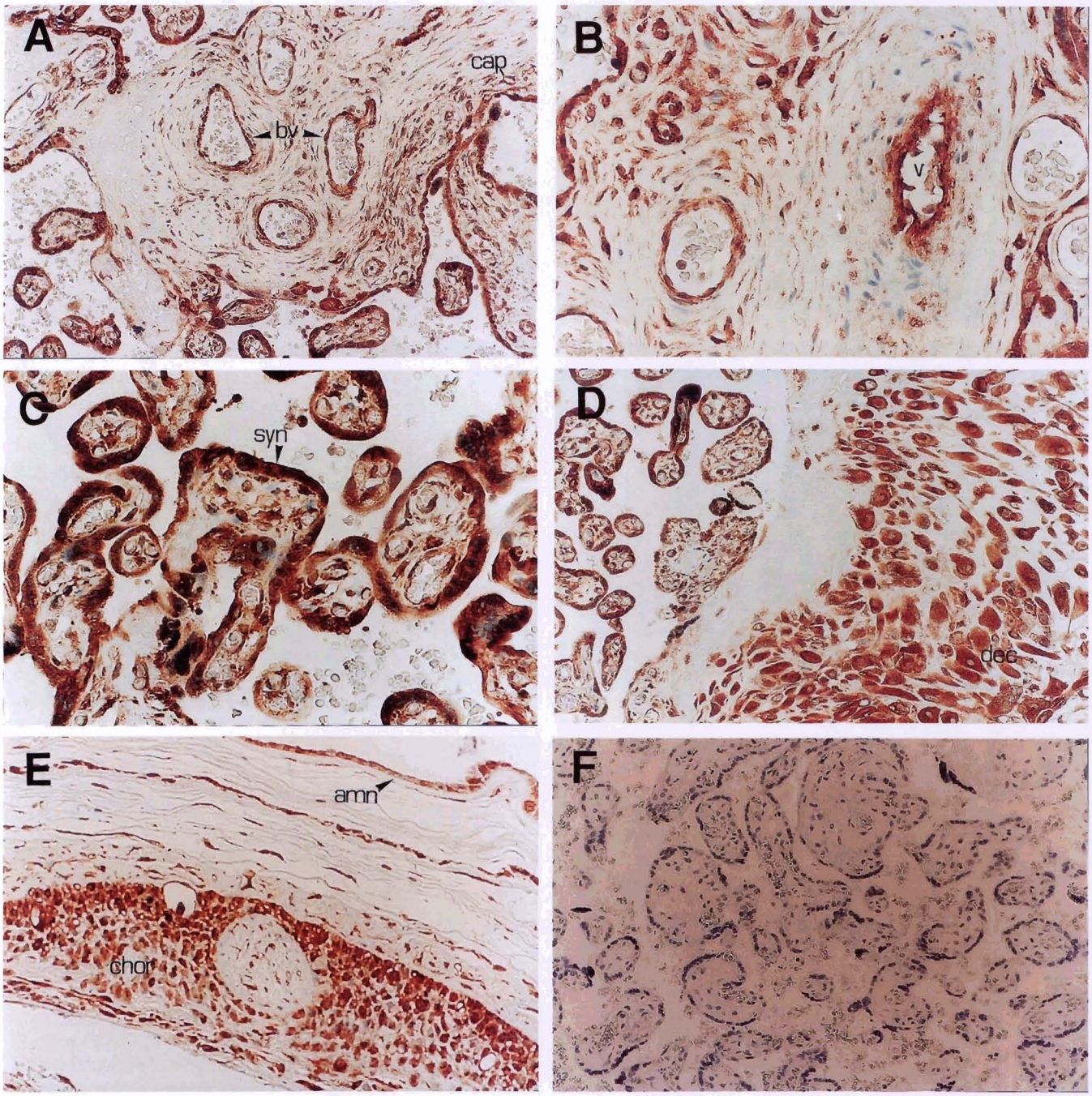


Fig. 3. VEGFR-3 immunostaining in normal human term placenta. **A.** Moderate immunostaining for VEGFR-3 is detected in the endothelial cells of the large vessels of the stem villi (bv) with weaker staining apparent in the smaller capillaries (cap). **B.** Intense immunostaining for VEGFR-3 localises to the surrounding venous tissues (v). **C.** The syncytiotrophoblast of the terminal villi show intense immunostaining for VEGFR-3 (syn). **D.** The maternal decidua demonstrates strong staining for VEGFR-3 (dec) There is no staining of the extra villous trophoblast identified by strong cytokeratin immunostaining (not shown here). **E.** Strong immunostaining for VEGFR-3 in the avascular amnion (amn) and vascular chorion (chor). **F.** Control sections incubated with non specific goat serum show no immunostaining. A, D, F, x 280; B, C, x 560

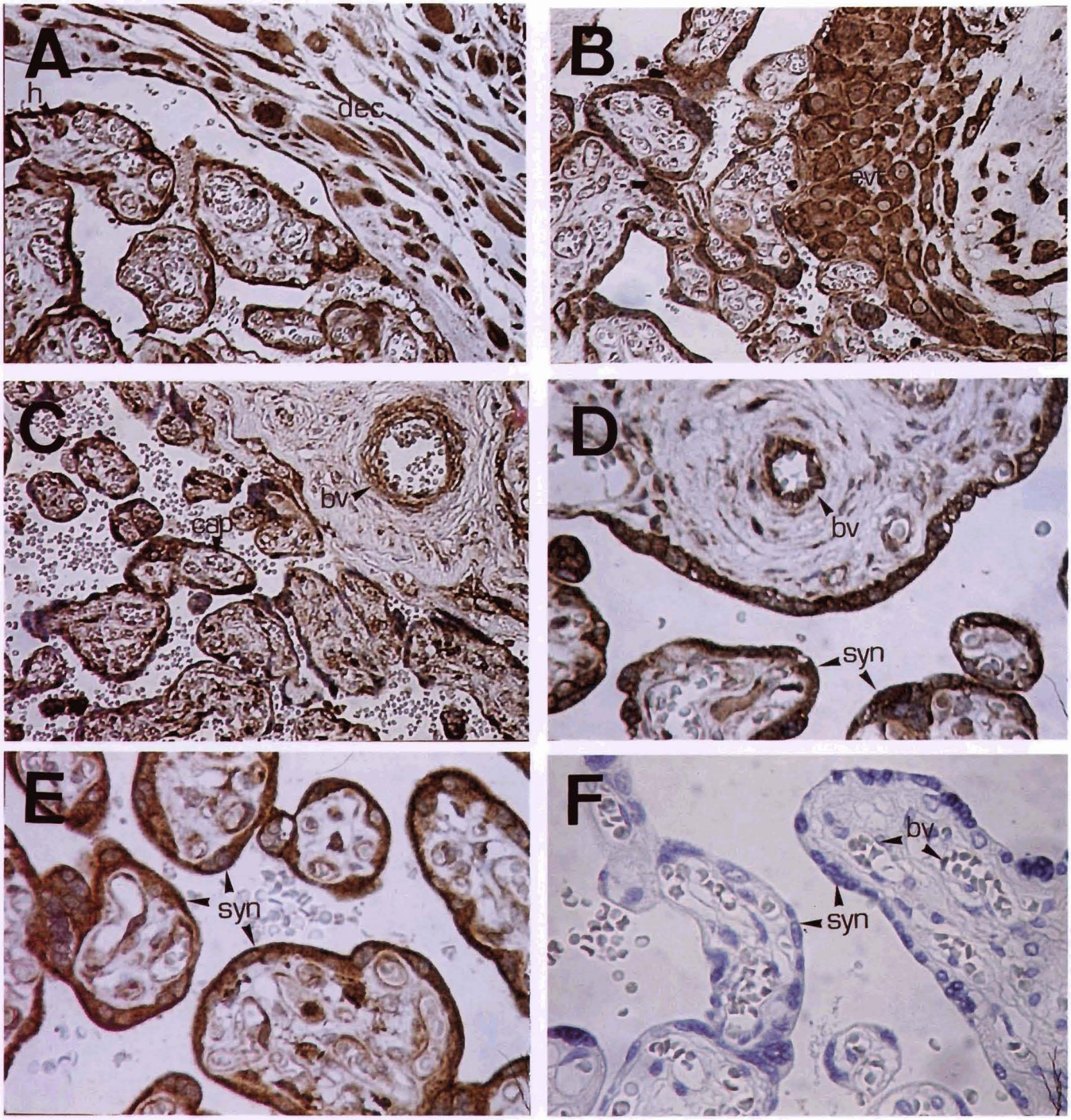


Fig. 4. VEGFR-2 immunostaining in placentae from normal and preeclamptic and IUGR pregnancy. (A-C) 38 weeks gestation. **A.** Very intense immunostaining for VEGFR-2 is demonstrated in the vasculosyncytial membrane, maternal decidual cells (dec) and fetal hoefbauer cells (h). **B.** Intense immunostaining for VEGFR-2 is also detected in the extravillous trophoblast (evt) identified by cytokeratin immunostaining (not shown here). **C.** Vascular endothelial cells of blood vessels (bv) within placental villi show moderate immunostaining for VEGFR-2. **D and E.** There is no change in immunostaining intensity or distribution for VEGFR-2 in PE (panel D, 36 week gestation) or IUGR (panel E 35 week gestation) sections. **F.** Control sections incubated without the primary antibody showed no immunostaining. A-C, x 280; D-F, x 560

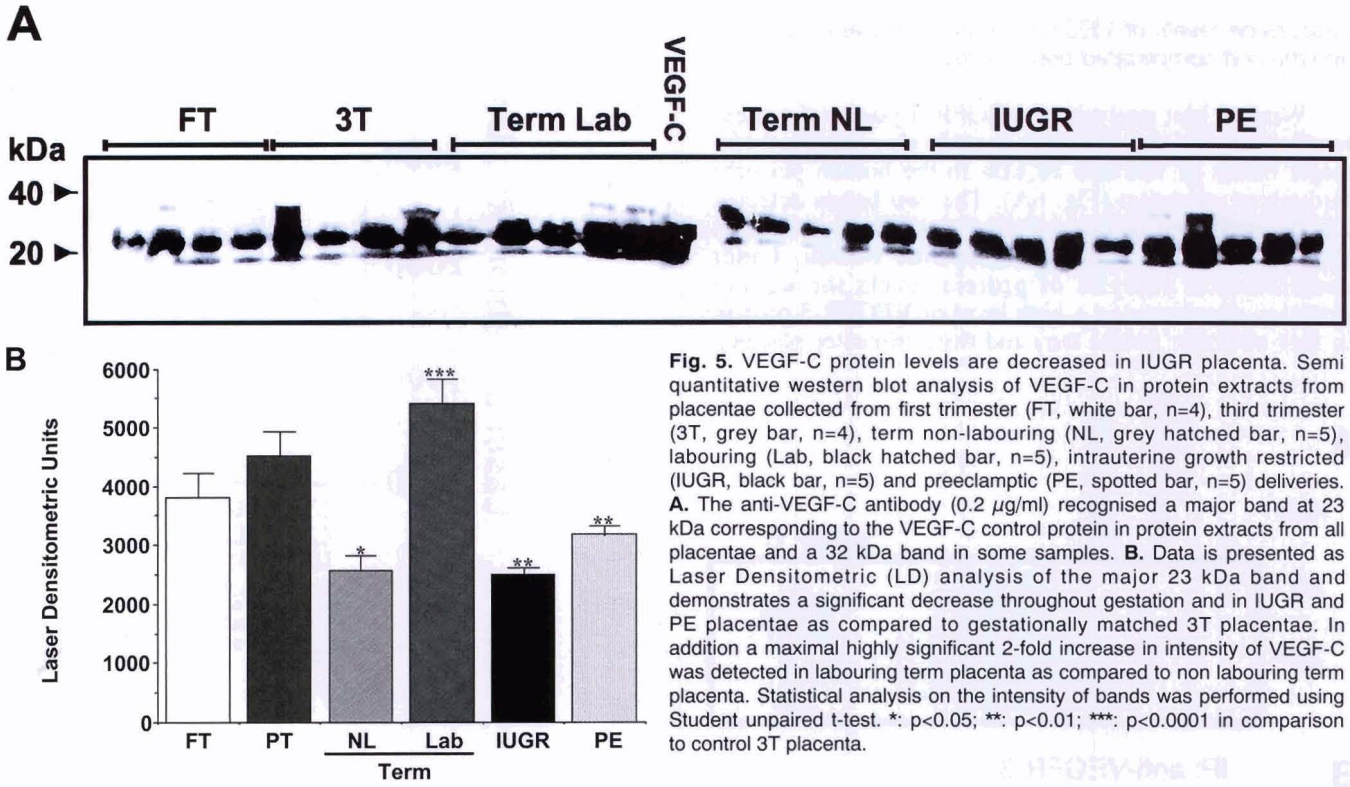


Fig. 5. VEGF-C protein levels are decreased in IUGR placenta. Semi quantitative western blot analysis of VEGF-C in protein extracts from placentae collected from first trimester (FT, white bar, n=4), third trimester (3T, grey bar, n=4), term non-labouring (NL, grey hatched bar, n=5), labouring (Lab, black hatched bar, n=5), intrauterine growth restricted (IUGR, black bar, n=5) and preeclamptic (PE, spotted bar, n=5) deliveries. **A.** The anti-VEGF-C antibody (0.2 μg/ml) recognised a major band at 23 kDa corresponding to the VEGF-C control protein in protein extracts from all placentae and a 32 kDa band in some samples. **B.** Data is presented as Laser Densitometric (LD) analysis of the major 23 kDa band and demonstrates a significant decrease throughout gestation and in IUGR and PE placentae as compared to gestationally matched 3T placentae. In addition a maximal highly significant 2-fold increase in intensity of VEGF-C was detected in labouring term placenta as compared to non labouring term placenta. Statistical analysis on the intensity of bands was performed using Student unpaired t-test. *: p<0.05; **: p<0.01; ***: p<0.0001 in comparison to control 3T placenta.

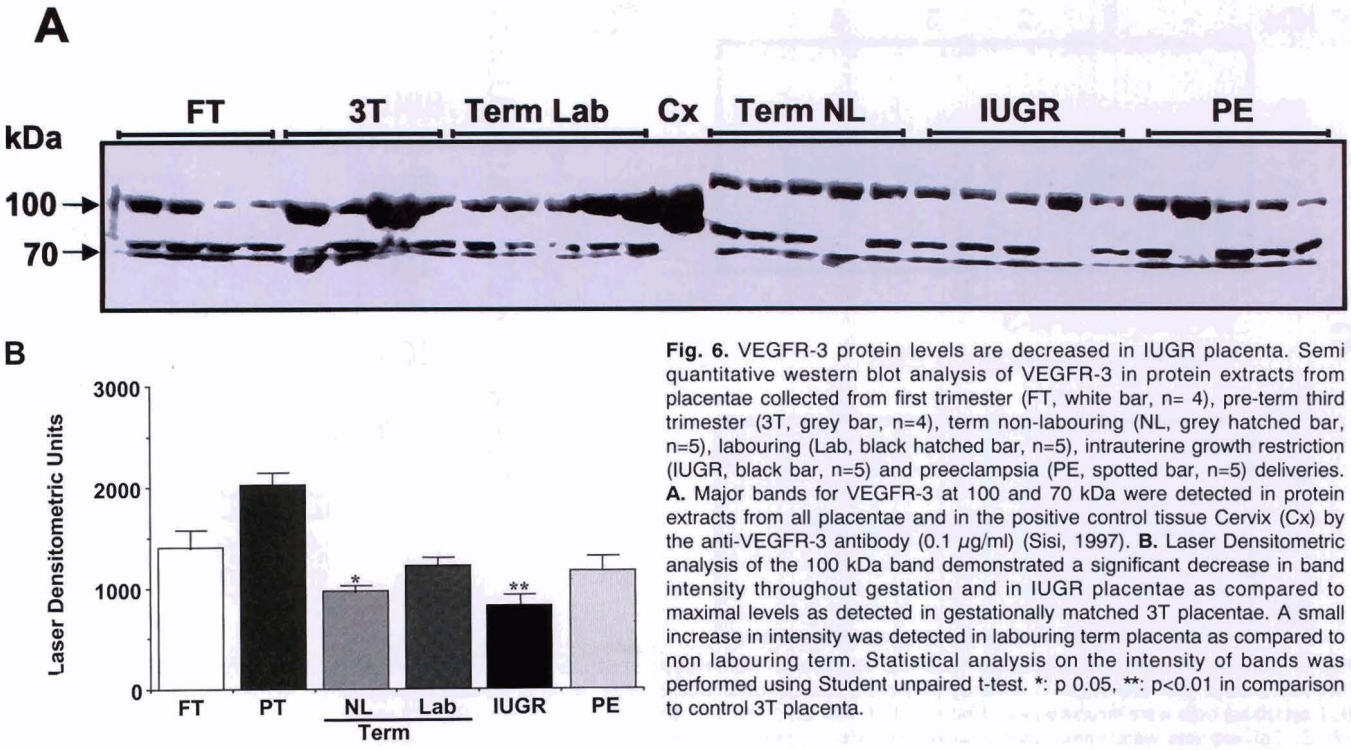
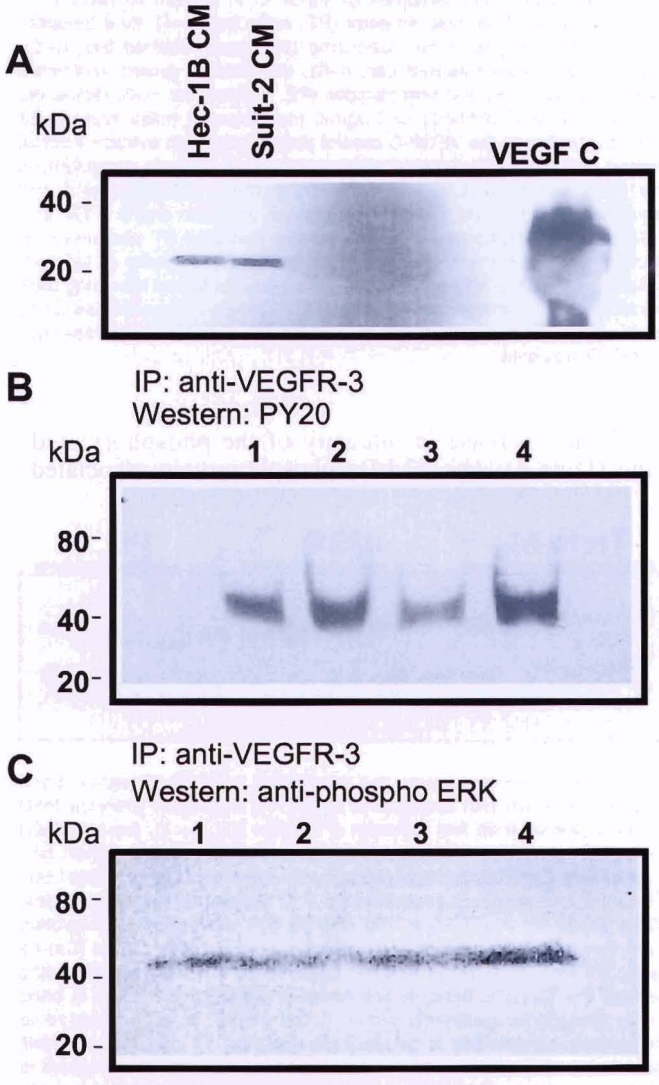


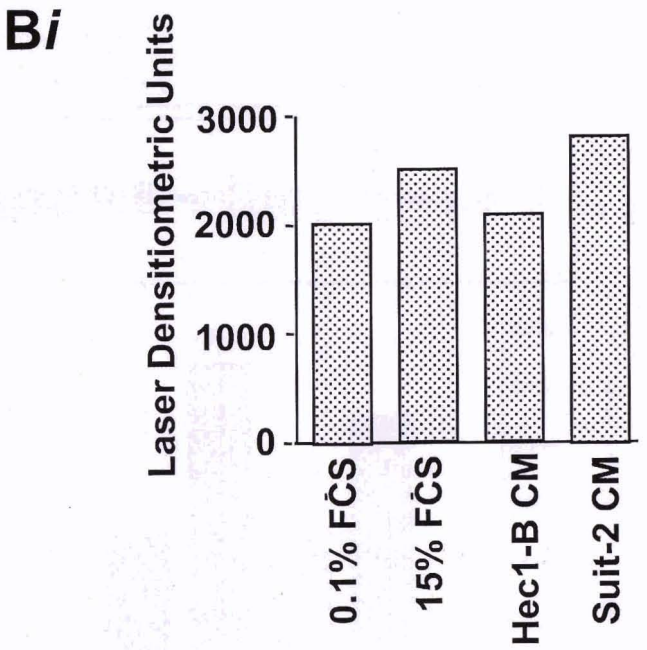
Fig. 6. VEGFR-3 protein levels are decreased in IUGR placenta. Semi quantitative western blot analysis of VEGFR-3 in protein extracts from placentae collected from first trimester (FT, white bar, n= 4), pre-term third trimester (3T, grey bar, n=4), term non-labouring (NL, grey hatched bar, n=5), labouring (Lab, black hatched bar, n=5), intrauterine growth restriction (IUGR, black bar, n=5) and preeclampsia (PE, spotted bar, n=5) deliveries. **A.** Major bands for VEGFR-3 at 100 and 70 kDa were detected in protein extracts from all placentae and in the positive control tissue Cervix (Cx) by the anti-VEGFR-3 antibody (0.1 μg/ml) (Sisi, 1997). **B.** Laser Densitometric analysis of the 100 kDa band demonstrated a significant decrease in band intensity throughout gestation and in IUGR placentae as compared to maximal levels as detected in gestationally matched 3T placentae. A small increase in intensity was detected in labouring term placenta as compared to non labouring term. Statistical analysis on the intensity of bands was performed using Student unpaired t-test. *: p 0.05, **: p<0.01 in comparison to control 3T placenta.

n=5, Fig. 5B). Placenta from both IUGR (solid bar) and pre-eclamptic (dotted bar) pregnancies demonstrated a significant decrease in VEGF-C protein levels as compared to gestationally matched t hird trimester placenta (p<0.001 vs 3T, n=5, Fig. 5B).

Expression levels of VEGFR-3 protein in placentas from healthy and complicated pregnancies

Western blot analysis of VEGFR-3 placental protein levels with the anti-VEGFR-3 antibody identified two major bands of 100 and 70 kDa in the human placenta throughout gestation (Fig. 6A). The two bands detected can be attributed to the reducing conditions present in the assay which reduce disulphide bonds. Laser densitometric analysis of protein levels throughout gestation demonstrated a high level of VEGFR-3 protein in first trimester (white bar) and third trimester placenta





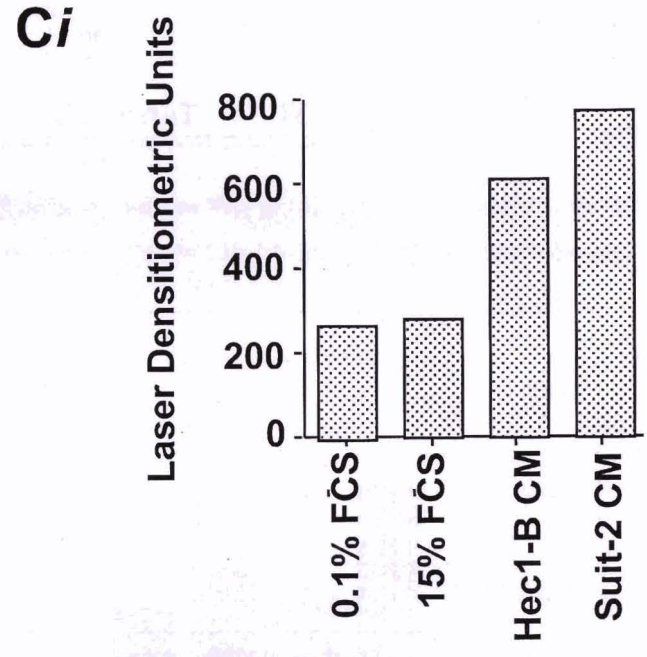


Fig. 7. Autophosphorylation of trophoblast VEGFR-3. **A.** Western blot analysis of tumour cell conditioned medium (CM) for the presence of VEGF-C. Blot was probed with anti-VEGF-C antibody (0.2μg/ml) and demonstrated a single band of 23 kDa corresponding to the control VEGF-C protein. **B.** Trophoblast cells were incubated with DMEM/F-12 0.1%FCS (Lane 1), 15%FCS (Lane 2) Hec-1B CM (Lane 3) or Suit-2 CM (Lane 4) for 2 hours at 37 °C. Cell extracts were immunoprecipitated with anti-VEGFR-3 antibody (1 μg/ml) overnight followed western blot analysis with PY20 anti-phosphotyrosine antibody (0.5 μg/ml). A single phosphorylated band of 42kDa was observed in all cases. Laser densitometric analysis demonstrated an increase in intensity of the 42 kDa band on addition of 15%FCS or Suit-2 CM. **C.** Identification of the 42 kDa band as phospho-ERK. The blot from panel B was stripped and reprobed with anti-phospho-ERK antibody (0.025 μg/ml) identifying the 42 kDa phospho-protein associated with VEGFR-3 immunoprecipitates as phosphorylated ERK. Laser densitometric analysis demonstrated a 189% increase in ERK band intensity on stimulation with Suit-2 CM.

(grey bar) (Fig. 6B). As gestation progresses to term VEGFR-3 protein levels show a significant decrease in expression ($p<0.05$, n=5). However, a small increase in labouring term placenta (black hatched bar) was observed, as compared to non labouring term placenta (grey hatched bar), obtained by elective caesarean section. Placenta from pregnancies complicated by IUGR (solid bar) displayed a significant decrease in VEGFR-3 protein levels, as compared to gestationally matched third trimester placenta ($p<0.01$ vs 3T, n=5) (Fig. 6B).

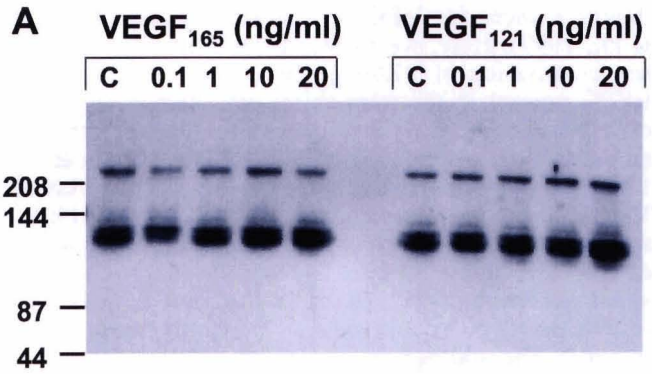


Fig. 8. Autophosphorylation of VEGFR-2 in human trophoblast. Stimulation's were initiated by the addition of increasing concentrations of 0.1-20 ng/ml $VEGF_{165}$ (Lanes 2-5) or $VEGF_{121}$ (Lanes 7-10) and a 2 hour incubation at 37 °C. Cell extracts were immunoprecipitated with anti-VEGFR-2 antibody (1 μg/ml) overnight followed western blot analysis with PY20 antiphosphotyrosine antibody. $VEGF_{165}$ has little effect above control levels of phosphorylated bands detected at 210, 130 and 120 kDa. $VEGF_{121}$ causes a dose dependant increase above control levels of the 210 and 120 kDa phosphorylated bands.

Activation of VEGFR-3 and association of phospho-ERK

In this study we further demonstrate the presence of functional VEGFR-3 in first trimester trophoblast (Fig. 7). As tumour cell lines were reported to secrete VEGF-C (Joukov et al., 1996) we first investigated the presence of VEGF-C in conditioned media (CM) from a human endometrial epithelial adenocarcinoma cell line, (Hec-1B) or a pancreatic carcinoma cell line, (Suit-2). Western blot analysis of the Hec-1B and Suit-2 CM displayed a single 23 kDa band corresponding to the control VEGF-C protein (Fig. 7A). Next, to investigate the activation of trophoblastic VEGFR-3 quiescent first trimester trophoblast ED_{27} cells were stimulated for 2 hours with conditioned media (CM) obtained from the carcinoma cell lines or 15% FCS. Cell lysates were immuno-precipitated with anti-VEGFR-3 antibody (0.5 μg/ml) and tyrosine kinase phosphorylation was assessed using a monoclonal anti-phosphotyrosine kinase antibody PY-20. A single phosphorylated band of 42 kDa associated with the VEGFR-3 in the control (Lane 1; 0.1% FCS) (Fig. 7B). On stimulation with 15% FCS (Lane 2) or conditioned media obtained from Suit-2 cells (Lane 4) (Fig. 7B,Bi) there was an increase in band intensity of 25% and 39% respectively as assessed by laser densitometric analysis. Hec-1B cell conditioned media caused no increase in intensity of the phosphorylated band (Lane 3). The 42 kDa phosphoprotein associated with VEGFR-3 immuno-precipitates was further identified by stripping and reprobing of the blot with a specific anti-phospho-ERK antibody demonstrating an

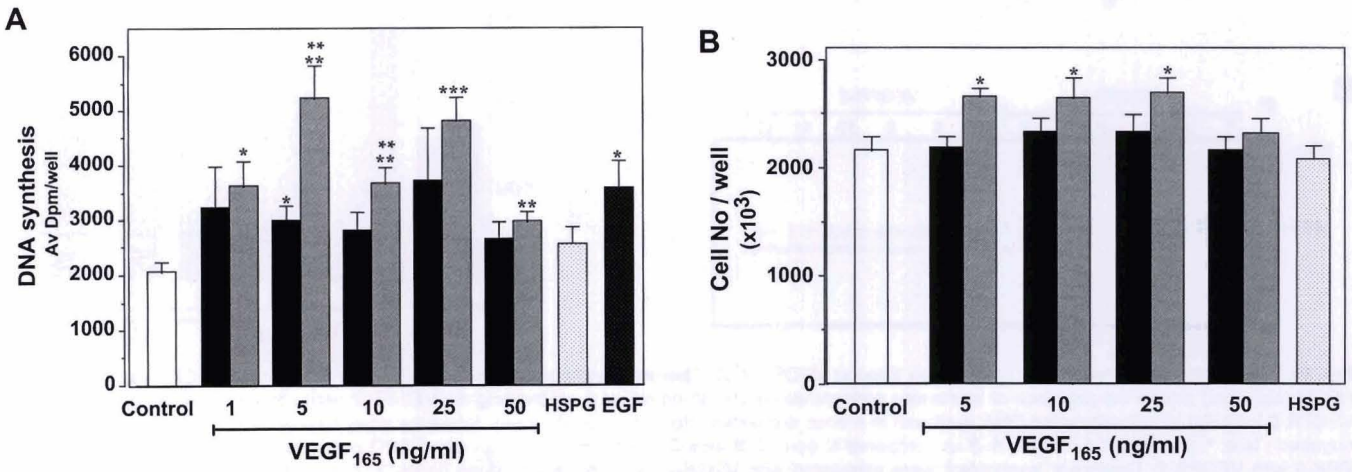


Fig. 9. Effect of $VEGF_{165}$ alone and premixed with HSPG on trophoblast proliferation. Quiescent trophoblast cells were incubated with $VEGF_{165}$ or $VEGF_{165}$+HSPG in increasing concentrations 1-50 ng/ml for 24 hours, and DNA synthesis and cell numbers determined as described in materials and methods. **A.** Addition of $VEGF_{165}$ alone (solid bars) had little effect on [^{3}H] thymidine incorporation causing a small significant increase ($p<0.05$, n=3) above control at 5 ng/ml alone and **B.** had no effect on cell number. **A.** Addition of $VEGF_{165}$ premixed with HSPG (hatched bars) caused a significant increase in [3H] thymidine incorporation at all doses with the maximal response observed at 5 ng/ml stimulating a 2.5 fold increase in DNA synthesis ($p<0.001$, n=3) **B:** and an increase in cell number ($p<0.05$, n=3). Addition of HSPG (40 ng/ml) had no effect on trophoblast DNA synthesis or cell number. Data is expressed as a mean±S.D. of three independant experiments performed in triplicate determinations per experiment. Statistical analysis was performed using the students unpaired t test. *: $p<0.05$; **: $p< 0.001$, n=3 as compared to control.

increase in band intensity on stimulation with Suit-2 CM (189%, Lane 4) (Fig. 7C,Ci). This band corresponds to the previously reported size of ERK-2 (review, Karin, 1995).

Tyrosine kinase activation of VEGFR-2 by $VEGF_{121}$ and $VEGF_{165}$

We further demonstrate the presence of functional VEGFR-2 receptor in the human first trimester trophoblast ED_{27}. Quiescent cells were stimulated with increasing concentrations of $VEGF_{121}$ or $VEGF_{165}$ (ng/ml) and cell lysates prepared. Following immunoprecipitation with anti-VEGFR-2 antibody, tyrosine kinase phosphorylation was assessed as above. VEGFR-2 immunoprecipitates display major phosphorylated bands detected at 210 kDa, 130, and 120 kDa that were present in both control and stimulated cells (Fig. 8). Addition of increasing concentrations of $VEGF_{121}$ induced a dose-dependent increase in the phosphorylation of the 210 kDa VEGFR-2 with maximal phosphorylation observed at 20 ng/ml. In contrast stimulation of the cells with $VEGF_{165}$ had little effect on VEGFR-2 phosphorylation above control levels (Fig. 8).

$VEGF_{165}$ premixed with heparin sulphate proteoglycan (HSPG) stimulates trophoblast proliferation

We have previously reported that the addition of $VEGF_{165}$ to first trimester human trophoblast does not increase DNA synthesis in a reproducible manner though it increases VEGFR-1 phosphorylation (Ahmed et al., 1997). Here, we further investigate the potential sequesterisation of $VEGF_{165}$ by VEGFR-1 and native HSPG present in the trophoblast cell membrane or the extracellular matrix (ECM) as has been demonstrated in an extravillous trophoblast cell line (Athanassiades et al., 1998). This possibility was tested by premixing $VEGF_{165}$ with excess (1μg/ml) soluble HSPG's, saturating the heparin binding sites prior to stimulation of the cells and a 30 hour incubation at 37 °C. DNA synthesis was assessed over the last 6 hours of incubation by the addition of [^{3}H] thymidine (0.2 μCi/ml). The addition of increasing doses of $VEGF_{165}$ alone (solid bars) stimulated a small increase in [^{3}H] thymidine incorporation above basal control levels (white bar). In all cases, $VEGF_{165}$ mediated DNA synthesis was below that of epidermal growth factor (2

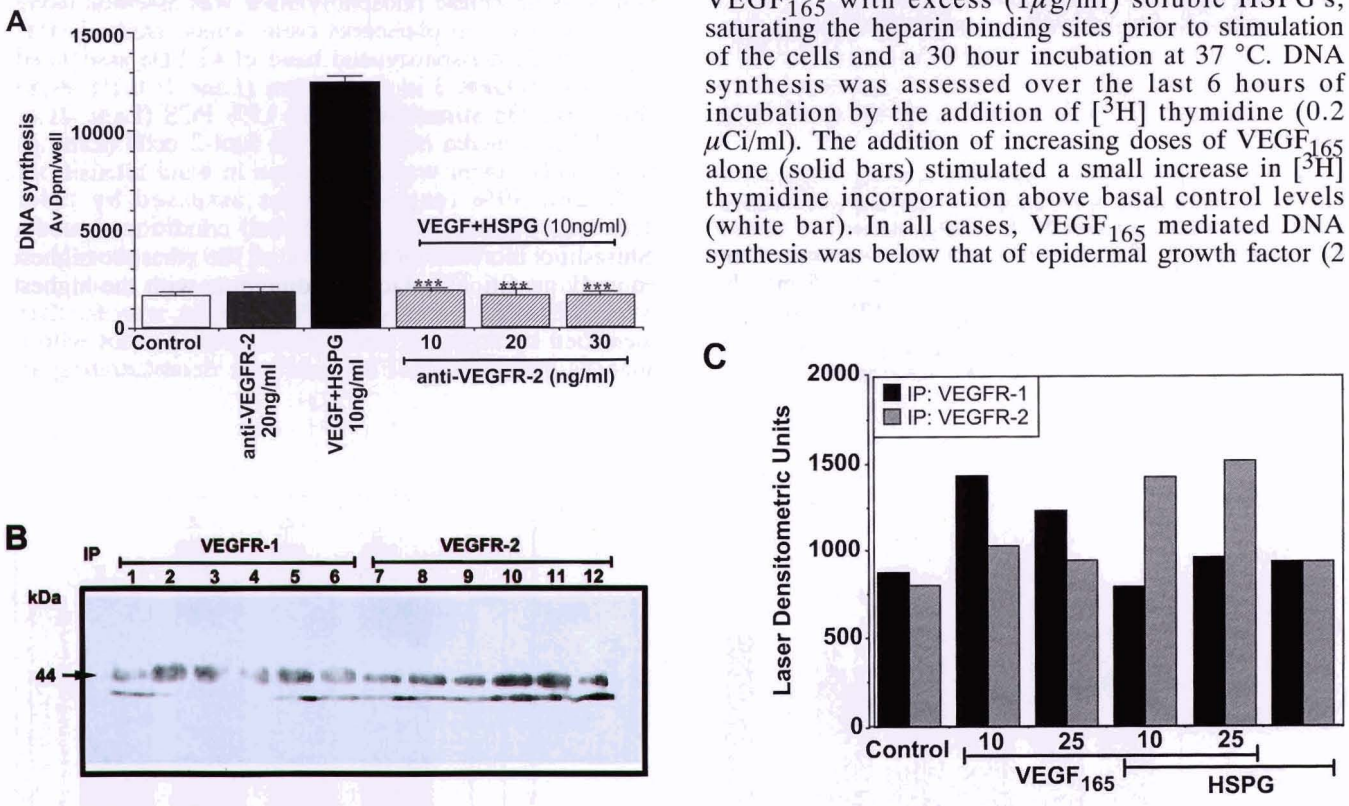


Fig. 10. VEGF stimulates trophoblast proliferation through VEGFR-2. **A.** Quiescent trophoblast were stimulated with 10 ng/ml $VEGF_{165}$ premixed with HSPG (solid bar) and in the presence of increasing concentrations (10-30 ng/ml) of a neutralising antiVEGFR-2 antibody (hatched bars). Blockage of VEGFR-2 inhibited VEGF stimulated DNA synthesis in a dose dependant manner ($p<0.001$, n=3). Statistical analysis was performed using the students unpaired t test. *: $p<0.05$; **: $p< 0.001$, n=3 as compared to control. **B and C.** Premixing $VEGF_{165}$ with HSPG potentiates the association of phospho-ERK-2 with VEGFR-2. Quiescent trophoblast were stimulated with $VEGF_{165}$ (10-25 ng/ml), alone (lanes 2, 3, 8 and 9, B) and in the presence of excess HSPG (lanes 4, 5, 10, and 11 B) as in Fig 9. HSPG alone (40 ng/ml) was used as a control (lanes 6 and 12). Incubations were carried out for 2 hours at 37 °C prior to extraction of total cell proteins and immunoprecipitation with either anti-VEGFR-1 (lanes 1-6) or anti-VEGFR-2 (lanes 7-12) antibodies as described in Materials and methods. B: The association of activated ERK with VEGFR-1 (lanes 1-6) or VEGFR-2 (lanes 7-12) was assessed by immunoblotting with a polyclonal anti-phospho ERK antibody (0.025 μg/ml) demonstrating a specific 42 kDa band in all samples. C: Laser Densitometric analysis of the 42 kDa band demonstrated an increase in phospho-ERK-2 in VEGFR-1 immunoprecipitates (solid bars) on stimulation with $VEGF_{165}$ alone (lanes 2 and 3 A). In contrast VEGFR-2 immunoprecipitates (hatched bars) demonstrate an increase in ERK band intensity on stimulation with $VEGF_{165}$ in the presence of HSPG (lanes 10 and 11 A). Data shown is representative of three independant experiments each showing a similar profile.

ng/ml, black spotted bar) and variability between experiments high (Fig. 9A). In contrast, $VEGF_{165}$ premixed with HSPG (hatched bars) significantly stimulated [^{3}H] thymidine incorporation in a dose-dependant manner (Fig. 9A) while HSPG alone (40 ng/ml) had no effect. Maximal stimulation was observed with 5 ng/ml $VEGF_{165}$ plus HSPG causing a 2.5 fold increase above control ($p<0.001$ n=3). To assess that the observed increase in DNA synthesis was due to cell replication and not DNA repair, cell numbers were assessed over 48 hours under the same stimulatory conditions. $VEGF_{165}$ (5-25 ng/ml) in the presence of HSPG stimulated a significant increase in trophoblast cell number ($p<0.01$, n=3) (Fig. 9B).

HSPG potentiates $VEGF_{165}$ mediated trophoblast proliferation via VEGFR-2 and increased association of phospho-ERK

Quiescent first trimester trophoblast cells were stimulated with 10 ng/ml $VEGF_{165}$ premixed with HSPG (solid bar) alone, and in the presence of increasing concentrations of a neutralising anti-VEGFR-2 antibody (ng/ml) (hatched bars) and DNA synthesis assessed as above. Addition of $VEGF_{165}$ plus HSPG stimulated an increase in incorporated [^{3}H] thymidine in a manner that was attenuated to control levels by neutralisation of VEGFR-2 ($p<0.001$, n=3, Fig. 10A). Potentiation of the $VEGF_{165}$-VEGFR-2 downstream signalling pathway by HSPG was further confirmed by western blotting of VEGFR-1 and VEGFR-2 immunoprecipitates with the anti-phospho-ERK antibody. Western blot analysis of phosphorylated ERK associated with VEGFR-1 or VEGFR-2 in trophoblast stimulated with $VEGF_{165}$ alone, (solid bars) or in combination with HSPG (hatched bars) demonstrated a specific 42 kDa band in all samples (Fig. 10B). Interestingly, incubation with $VEGF_{165}$ alone mediated an increase in phospho-ERK associated with VEGFR-1 immunoprecipitates, stimulating an average 1.62 fold increase in intensity of the 42 kDa ERK-2 band (lanes 2, 3) (Fig. 10B,C). In VEGFR-2 immunoprecipitates the 42 kDa ERK-2 band showed no increase above control on stimulation with $VEGF_{165}$ alone (lanes 7 and 8) (Fig. 10B,C). In contrast, premixing $VEGF_{165}$ with HSPG prior to stimulation of the trophoblast with equivalent $VEGF_{165}$ concentration attenuated the intensity of the 42 kDa band associated with VEGFR-1 to control levels (lanes 4 and 5 as compared to lane 1: Fig. 10B,C). While in VEGFR-2 immunoprecipitates the 42 kDa ERK-2 band demonstrated a 1.84 fold increase in intensity in samples stimulated with $VEGF_{165}$ in the presence of HSPG (lanes 10 and 11: Fig. 10B,C). Incubation of the trophoblast with HSPG 40 ng/ml had no stimulatory effect on association of ERK above control levels in either VEGFR-1 (lane 6: Fig. 10B) or VEGFR-2 immunoprecipitates (lane 12: Fig. 10B). Thus this study conclusively demonstrates that saturation of the VEGFR-1/HSPG binding domain of $VEGF_{165}$ allows direct interaction of this isoform with VEGFR-2 potentiating association of activated ERK to stimulate significant trophoblast DNA synthesis.

Discussion

This study provides additional evidence for an autocrine role for the VEGF family in trophoblast function from its co-localisation of VEGF-C, VEGFR-2 and VEGFR-3 to the trophoblast in human placenta. We show using *in vitro* studies that trophoblast express functional VEGFR-2 and VEGFR-3 that on activation stimulate trophoblast proliferation and increase the association of phospho-ERK. Furthermore, the expression of VEGF-C and VEGFR-3 were decreased in placenta from pregnancy complicated by IUGR lending support to our central hypothesis that a disturbance in relative levels of a number of critical growth factors may contribute to the observed aged syncytium and poor angiogenesis in severe IUGR.

The first two trimesters of placental development are dominated by the establishment and formation of the richly branched capillary beds of the mesenchymal and immature intermediate villi of the placenta (Kaufmann et al., 1985; Leiser et al., 1985; Demir et al., 1989). The development of the villous angioarchitecture appears to correlate with the expression levels of VEGFR-2 (Vuckovic et al., 1996) and VEGF (Jackson et al., 1994; Shirashi et al., 1996; Khaliq et al., 1999) that are highest in early gestational placenta coinciding with the highest levels of cellular proliferation and angiogenesis. This study similarly demonstrates that high levels of VEGF-C and VEGFR-3 proteins were detected during early placental development suggesting an active role of VEGF-C in placental angiogenesis and villous development. In contrast, PlGF (Khaliq et al., 1999) and VEGFR-1 (Ahmed et al., 1995; Clark et al., 1996) levels are higher during late placental development, dominated by non-branching angiogenesis during the formation of the mature intermediate and terminal villi.

Interestingly in the term placenta both VEGF-C and VEGFR-3 proteins localised with highest intensity to the syncytiotrophoblast membrane of the terminal villi, the site of transport between maternal and fetal circulations. The lymphatic endothelium is similarly a highly permeable transport tissue to which VEGFR-3 expression becomes restricted to during embryonic development (Kaipainen et al., 1995). We suggest that VEGF-C and VEGFR-3 may play a similar role in trophoblast in mediating transport between the maternal and fetal circulations. We have previously demonstrated that trophoblast express both VEGF and its receptor VEGFR-1 (Ahmed et al., 1995). Addition of exogenous $VEGF_{165}$ stimulates the release of nitric oxide via VEGFR-1 from a first trimester trophoblast cell line (Ahmed et al., 1997). In this study, the co-localisation of VEGF-C and both VEGFR-2 and VEGFR-3 to the terminally differentiated syncytiotrophoblast membrane lends further support to our hypothesis of an endothelial-

like function for the syncytium (Ahmed et al., 1995, 1997). This suggests that like VEGF, VEGF-C may play an autocrine role in trophoblast cell function, in addition, to its paracrine effects on endothelial cells.

In endothelial cells, both VEGFR-2 (Waltenberger et al., 1994) and VEGFR-3 (Joukov et al., 1996; Oh et al., 1997) were demonstrated to mediate cell proliferation and migration in response to VEGF and VEGF-C respectively. The localisation of VEGFR-2 in the invasive extravillous trophoblast suggests that VEGF-C expressed by the maternal decidua may act as a chemo-attractant for the invasive extravillous trophoblast during establishment of the intervillous utero-placental circulation. The expression of VEGFR-2 protein in trophoblast of the human placenta is supported by studies on mice. The homologous receptor Flk-1 is strongly expressed by trophoblast in midgestation mice (Millauer et al., 1993). Previously studies on human placental sections reported that VEGFR-2 was restricted to fetoplacental endothelial cells (Vuckovic et al., 1996). In the present study unmasking of the antigen, by microwave technique, was required to detect VEGFR-2 protein. Vuckovic and colleagues did not use this procedure which explains their inability to detect trophoblast VEGFR-2. Moreover, mRNA encoding VEGFR-2 detected by RT-PCR has been reported in choriocarinoma cell line BeWo (Charnock Jones et al., 1994), spontaneously transformed first trimester trophoblast ED_{27} (Ahmed et al., 1997) and in an extravillous trophoblast cell line (Athanassiades et al., 1998).

Severe IUGR is characterised by a small sized placenta with abnormal morphology, demonstrating poor angiogenesis, increased syncytial formation, and erythrocyte congestion of the terminal villi (Macara et al., 1996; Krebs et al., 1996) and suggested that there is "placental hyperoxia" in IUGR (Kingdom and Kaufmann, 1997). In this study, we demonstrate that both VEGF-C and VEGFR-3 proteins are significantly reduced in severe IUGR placenta as compared to levels detected in gestationally matched third trimester placenta. Interestingly, like VEGF, VEGF-C was reported to be up-regulated during retinal ischaemia (Simpson et al., 1999). Similarly, angiopoetin-2 was shown to be up-regulated by hypoxia in bovine microvascular endothelial cells (Oh et al., 1999) and is also decreased in severe IUGR (Dunk et al., 2000). These results are in agreement with our earlier finding that PlGF levels are increased in IUGR and up-regulated by hyperoxia (Khaliq et al., 1999). Although these studies do not prove a direct causative link with abnormal villous development, collectively these data support the hypothesis of "placental hyperoxia" in severe IUGR. The strong association between oxygen levels and changes in the expression of these growth factors lends support to our hypothesis that increased oxygen levels surrounding the placental villi may limit angiogenesis as a result of disturbances in the expression and function of oxygen-sensitive angiogenic growth factors.

Interestingly, levels of VEGF-C protein were found to be 2-fold higher in placenta obtained from labouring compared with non-labouring deliveries suggesting a potential role of VEGF-C in parturition. Moreover, VEGF-C mRNA is known to be upregulated by the proinflammatory cytokine IL-1ß (Ristimaki et al., 1997) and this cytokine plays a role in the initiation of labour (Kelly et al., 1996). Indeed the related factor VEGF has been demonstrated to promote parturition (Sisi et al, 1996). However as delivery causes hypoxia and other changes to the placenta it is not possible to draw a firm conclusion about the role of VEGF-C in the initiation of parturition without further studies.

Tyrosine kinase receptors have been demonstrated to mediate DNA synthesis and cell proliferation through many signalling molecules that physically interact with and become activated by the receptors. Several of these signal transduction molecules act through the Ras and downstream MAP kinase pathways while others are ras-independant (Roche et al., 1996). In particular the activation and phosphorylation of the 42/44 kDa MAP kinase ERK-1/2 is associated with cellular proliferation (review Karin, 1995). This study demonstrated that stimulation of the trophoblast with CM containing VEGF-C caused an increase in phospho-ERK-2 associated with VEGFR-3 immunoprecipitates. As stated earlier VEGF-C has been demonstrated to stimulate lymphatic endothelial cell proliferation (Fitz et al., 1997). This data suggests that activation of VEGFR-3 by VEGF-C may mediate trophoblast proliferation in addition to its effects on endothelium in the human placenta. However, as the VEGF-C protein is not yet commercially available, the physiological role of VEGF-C in the human placenta and trophoblast cell function remains to be fully elucidated.

It is known that $VEGF_{165}$ binds to both VEGFR-1 and VEGFR-2, but with higher affinity to VEGFR-1 as it contains a 44 amino acid heparin binding domain encoded by exon seven (Terman et al., 1994). In contrast, $VEGF_{121}$ binds to VEGFR-2 alone as it lacks the 110-165 heparin/VEGFR-1 binding domain (Gitay-Goren et al., 1996; Keyt et al., 1996a,b). The present study demonstrates that $VEGF_{121}$ stimulated a dose-dependant phosphorylation of the 210 kDa band in VEGFR-2 immunoprecipates from the first trimester trophoblast. The 210 kDa band detected represents direct auto-phosphorylation of VEGFR-2, and is in agreement with molecular weights reported previously for autophosphorylation (Waltenberger et al., 1994; Thieme et al., 1995) and receptor-ligand cross-linking studies (Thieme et al., 1995; Gitay-Goren et al., 1996). The observed increase in phosphorylation of the 210 kDa VEGFR-2 band at all concentrations of $VEGF_{121}$ represents direct interaction of this isoform with VEGFR-2 while $VEGF_{165}$ had little effect. It has been proposed that the dose dependancy of $VEGF_{165}$ interaction with VEGFR-2 in trophoblast cells may be influenced by the competitive binding with VEGFR-1 or

cell surface HSPG (Athanassiaides et al., 1998). In particular heparin is demonstrated to potentiate the binding of VEGF to the extracellular domains of soluble or endothelial cell associated KDR/Flk-1 (Tessler et al., 1994; Roeckl et al., 1998). Moreover, $VEGF_{165}$ is thought to mediate endothelial cell proliferation through specific activation of VEGFR-2 (Waltenberger et al., 1994). This study demonstrates that premixing VEGF with HSPG stimulated a significant increase in trophoblast proliferation and increased association of phospho-ERK with VEGFR-2 immunoprecipitates. Moreover, neutralisation of the VEGFR-2 receptor inhibited $VEGF_{165}$ mediated trophoblast DNA synthesis to control levels. Thus, this study conclusively demonstrates that VEGF stimulated trophoblast proliferation via activation of VEGFR-2, as in endothelial cells.

$VEGF_{121}$ also stimulated an increase of a 120 and 130 kDa phospho-protein associated with VEGFR-2. In addition to direct autophosphorylation of its receptors, VEGF is known to induce the tyrosine phosphorylation of a variety of intracellular mediators of signal transduction containing src homology 2 (SH2) domains (Waltenberger et al., 1994; Guo et al., 1995; Landgren et al., 1998). Among these, a 120 kDa Ras GTPase (RasGAP) is thought to be important in mediating endothelial cell chemotaxis (Kundra et al., 1994), suggesting that VEGF may mediate trophoblast function via a GAP-mediated mechanism. The 130 kDa phosphorylated protein associated with VEGFR-2 immunoprecipitates is as yet unidentified, though other studies have demonstrated the common association of this band with VEGFR-1 on stimulation with both VEGF and PlGF, and VEGFR-2 on stimulation with VEGF (Landgren et al., 1998).

This study demonstrates the co-localisation of VEGFR-2 and VEGFR-3 and their novel ligand VEGF-C in placental cells of both non-endothelial and endothelial types suggesting a dual autocrine and paracrine role for VEGF family during placental angiogenesis and villous development. In support of an autocrine role, we have demonstrated the presence of functional VEGFR-2 and VEGFR-3 in first trimester human trophoblast by linking the respective receptor signalling to activation and association of the ERK-2 phospho-protein and cell proliferation. Moreover, this study provides further evidence of abnormal growth factor expression in severe IUGR placenta and its's association with placental hyperoxia. Both VEGF-C and VEGFR-3 protein levels were significantly decreased in severe IUGR and thus may contribute to the disturbances in trophoblast and endothelial cell proliferation and function. In conclusion, this data together with earlier studies (Ahmed and Kilby, 1997; Khaliq et al., 1999; Dunk et al., 2000) indicates that increased oxygen levels surrounding the placental villi may limit angiogenesis and villous development as a result of disturbances in the expression and function of oxygen-sensitive angiogenic growth factors.

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