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Role of VEGF Receptor-1 (Flt-1) in Mediating Calcium-Dependent Nitric Oxide Release and Limiting DNA Synthesis in Human Trophoblast Cells

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SUMMARY: Vascular endothelial growth factor (VEGF) receptor KDR (kinase-insert-domain–containing receptor) is linked to endothelial cell proliferation, and VEGF receptor Flt-1 (*fms*-like tyrosine kinase) is essential for the organization of embryonic vasculature. Flt-1 is also known to be expressed on adult endothelial and trophoblast cells, although its function has not yet been established. Herein we report that human trophoblast and endothelial cells contain functional Flt-1 receptors for VEGF that trigger the synthesis and release of nitric oxide (NO) by the activation of constitutive NO synthase (cNOS). In first-trimester human trophoblast cells isolated by chorionic villous sampling, $VEGF_{165}$ stimulated NO release in a concentration- and time-dependent manner, with a maximal increase of 60% (in comparison to basal release levels) occurring within 30 minutes (basal: 1342 pmol/ml; VEGF (10 ng/ml): 2162 pmol/ml; $p < 0.001$), as measured by an NO chemiluminescence analyzer. $VEGF_{20}$, a peptide fragment that is composed of the first 20 amino acids at N-terminus, displayed properties of a partial agonist. $VEGF_{165}$- and $VEGF_{20}$-mediated NO biosynthesis was attenuated by N^G-nitro-L-arginine in a concentration-dependent fashion, indicating NOS activation. VEGF-neutralizing anti-VEGF monoclonal antibody significantly inhibited VEGF-mediated NO release ($p < 0.001$), and the addition of a neutralizing anti–Flt-1 antibody inhibited the response by 79.6% ± 7.59%, an effect found to be reversible with higher concentrations of VEGF. In contrast, anti-KDR antibody had no significant inhibitory effect. RT-PCR confirmed the presence of mRNA encoding the Flt-1 and KDR receptors as well as the endothelial form of cNOS in trophoblast cells. $VEGF_{165}$-stimulated NO release was inhibited by genistein (5 μM; $p < 0.001$) as well as by the removal of calcium from the extracellular environment ($p < 0.001$), which suggests the contingency of this process on tyrosine phosphorylation and extracellular calcium, respectively. Addition of sodium nitroprusside, an NO donor, inhibited trophoblast DNA synthesis in a concentration-dependent manner, as measured by [^{3}H]thymidine incorporation, without affecting cell viability. VEGF under maximal NO production had no mitogenic activity, suggesting that trophoblast-derived NO may limit trophoblast proliferation. Endogenous trophoblast DNA synthesis increased 3-fold in the presence of anti--Flt-1 antibody but not in the presence of anti-KDR antibody, suggesting that Flt-1 functions as a growth suppressive receptor to counteract the proliferative actions of KDR. Levels of immunoreactive endothelial cNOS were markedly increased in growth-restricted placentae ($n = 4$) in comparison to those of normal ($n = 5$) placentae, which may account for the relatively small-sized placentae associated with intrauterine growth restriction. $VEGF_{165}$ stimulated NO release via phosphorylation of the Flt-1 receptor, indicating that VEGF may be an autocrine regulator of NO biosynthesis by aiding trophoblast penetration into spinal arterioles during the first trimester and preventing platelet aggregation within the placenta. Finally, the activation of Flt-1 receptor suppressed trophoblast DNA synthesis within the placenta via NO. (*Lab Invest 1997, 76:779–791*).

Extensive angiogenesis is required to establish the vascular structures that are necessary to the efficient transplacental transport of oxygen and nutrients from the mother to the fetus. This is achieved by growth of the villous tree and by alterations to the villous trophoblasts that line the maternal blood space and the fetal blood vessels within the villi (Benirschke and Kaufmann, 1995; Schlafke and Enders, 1975). Human placenta is a rich source of angiogenic growth factors and their receptors (Ahmed et al, 1995; Charnock-Jones et al, 1994; Ferriani et al, 1994; Khaliq et al, 1996b; Kilby et al, 1996; Shams and Ahmed, 1994; Sharkey et al, 1994), which are believed

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to control trophoblast function as well as stimulate vascular changes in both the spiral arteries supplying the placental bed and the placenta itself. Vascular endothelial growth factor (VEGF) was originally identified as a potent vascular permeability factor (Connolly et al, 1989; Sanger et al, 1983) or vasculotropin (Favard et al, 1991); it is composed of two identical subunits covalently linked by disulfide bonds. VEGF exhibits a low (18%) but significant overall sequence homology with platelet-derived growth factor (Ferrara et al, 1992) and a much closer (53%) homology with placenta growth factor (Maglione et al, 1991). Five different VEGF transcripts encoding polypeptides of 206, 189, 145, 165, and 121 amino acids are expressed by human cells (for review, see Dvorak et al, 1995). $VEGF_{165}$ is expressed predominantly in most instances, but whereas $VEGF_{121}$ and $VEGF_{165}$ are secreted in soluble form, the $VEGF_{189}$ and $VEGF_{206}$ remain cell-associated (Houck et al, 1992).

VEGF is a powerful multifunctional polypeptide that was initially thought to be a specific mitogen for endothelial cells (Ferrara et al, 1992); it has since been established, however, that it also mediates a number of other endothelial (Dvorak et al, 1995; Sanger et al, 1983) and nonendothelial (Ahmed et al, 1995; Charnock-Jones et al, 1994) effects. For example, VEGF evokes transient accumulation of cytoplasmic calcium and activation of both phospholipase C and D in human umbilical vascular endothelial (HUVE) cells (Seymour et al, 1996), unlike basic fibroblast growth factor, which only stimulates phospholipase D activity in these cells (Ahmed et al, 1994). VEGF binds to two phosphotyrosine kinase receptors with high affinity: Flt-1 (*fms*-like tyrosine kinase) and KDR (kinase-insert-domain–containing receptor) (Dvorak et al, 1995; Ferrara et al, 1992). The biologic activities observed both in vivo and in vitro appear to be mediated almost exclusively by the KDR receptor (Dvorak et al, 1995). In addition, both Flt-1 and KDR have been shown to undergo autophosphorylation upon binding of VEGF in transfected cells; and although Flt-1 was demonstrated to undergo a unique phosphorylation sequence upon binding of VEGF, its exact function in this process has yet to be fully elucidated (Waltenburger et al, 1994). These previous transfection studies of human Flt-1 or the KDR receptors in porcine aortic endothelial cells showed that VEGF stimulates chemotaxis and proliferation in KDR-transfected cells but not in Flt-transfected cells (Waltenburger et al, 1994). Recently reported null mutations in the equivalent mouse genes provide further data on the importance of the receptors in relation to endothelial cell biology. Inactivation of the mouse *flk*-1 gene (the equivalent of human KDR) is associated with a failure in endothelial cell development, an event that prevents the formation of an organized vascular system (Shalaby et al, 1995). The null mutation of the *flt* gene also results in fetal death, as is the case with *flk*-1, but leads to disorganized vascular endothelium from the earliest stages of development without affecting endothelial cell differentiation (Fong et al, 1995).

Flt-1, however, is also expressed on adult endothelial (Jakeman et al, 1992) and trophoblast cells, which are the first nonendothelial cells to be identified to express VEGF receptors (Ahmed et al, 1995; Charnock-Jones et al, 1994). In addition to its mitogenic and chemotactic abilities, VEGF induces fluid and protein extravasation from blood vessels (Dvorak et al, 1995) and mediates calcium influx (Brock et al, 1991; Seymour et al, 1996), raising the possibility that VEGF mediates the release of vasoactive factors such as nitric oxide (NO) into the local circulation. Formation of NO is achieved through the actions of three isoforms of NO synthase (NOS) catalyzing the conversion of L-arginine to L-citrulline plus NO in the presence of molecular oxygen (Lowenstein and Snyder, 1992). Neural and endothelial forms are constitutive (cNOS) and calcium-calmodulin–dependent, whereas the inducible enzyme is found in activated macrophages and vascular smooth muscle (Lowenstein and Snyder, 1992). Previous studies have localized the endothelial isoform of cNOS within the placenta (Conrad et al, 1993; Myatt et al, 1993), and implicated the important role of NO in maintaining low vascular tone in fetal placental vessels (Chaudhuri et al, 1993; Molnar et al, 1994). Herein, for the first time to date, we report that human trophoblast and vascular endothelial cells contain functional Flt-1 receptors for VEGF, which activate cNOS leading to the synthesis and release of nitric oxide.

Results and Discussion

RT-PCR analysis revealed that first-trimester trophoblast cells express the mRNA encoding Flt-1 and KDR (Fig. 1A). Immunocytochemical studies showed strong staining for VEGF (Fig. 1B) and Flt-1 (Fig. 1C), demonstrating the existence of an autocrine loop in trophoblast cells, unlike in endothelial cells (Dvorak et al, 1995; Ferrara et al, 1992)—although under-hypoxic stimuli endothelial cells also produce VEGF (Namiki et al, 1995). The immunoreactivity is consistent with in situ hybridization (Sharkey et al, 1994) and immunostaining (Ahmed et al, 1995) patterns in human placenta.

The most abundant product of the VEGF gene in most tissues appears to be $VEGF_{165}$ (Kim et al, 1992).

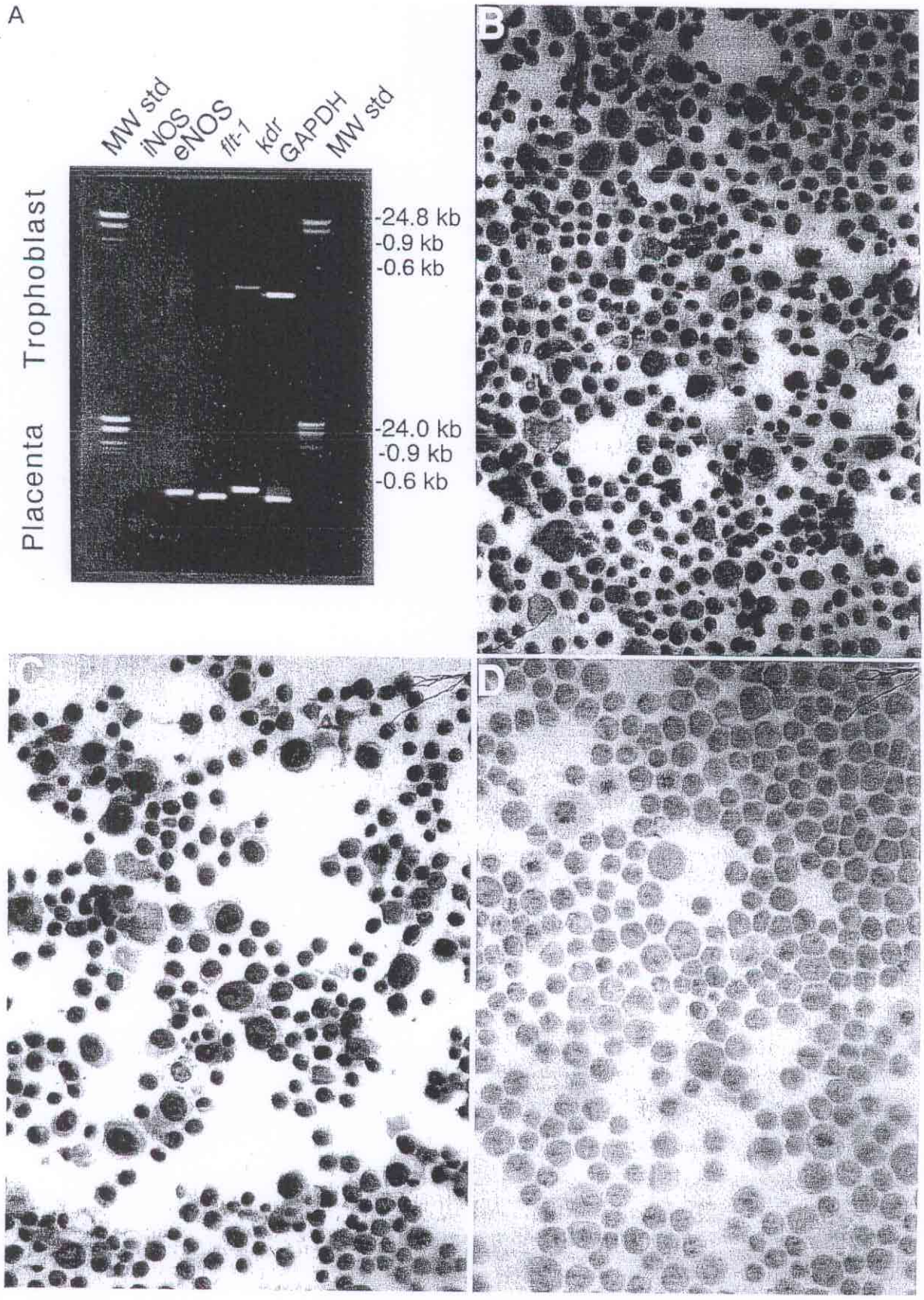

Figure 1.

Expression and localization of VEGF and its receptors in trophoblast cells. A, RT-PCR analysis of NOS and VEGF receptor subtypes in first trimester trophoblast cells and human placenta. MW std, Molecular weight standard. iNOS, Inducible NO synthase. eNOS, Endothelial NO synthase. Flt-1, *fms*-like tyrosine kinase. KDR, Tyrosine kinase domain receptor. Immunolocalization of VEGF (B) , Flt-1 (C), and negative control for Flt-1 (D) in trophoblast cells.

Incubation of confluent monolayer of trophoblast cells with $VEGF_{165}$ and its fragment (ie, $VEGF_{20}$, which is composed of the first 20 N-terminal amino acids of VEGF) resulted in the release of NO into the culture medium. Exposure of trophoblasts to 10 ng ml^{-1} $VEGF_{165}$ caused an increase in levels of NO release within 10 minutes, which peaked at 45 minutes (Fig. 2A). The addition of 50 ng/ml of VEGF-neutralizing monoclonal antibody (anti-VEGF mAb) after 35 minutes of 10 ng/ml $VEGF_{165}$ stimulation completely inhibited $VEGF_{165}$-evoked NO release within 35 minutes (Fig. 1A), indicating that $VEGF_{165}$-mediated NO release appears to be sustained over a 45-minute time period. In contrast, the addition of 5 ng ml^{-1} of the fragment, $VEGF_{20}$, caused no significant increase in NO release in the first 20 minutes; however, after 30 minutes of incubation, levels reached 89.7% ± 7.25% of baseline. Release of NO in response to both $VEGF_{165}$ and $VEGF_{20}$ appeared to be dose-dependent (Fig. 2B), although optimal response was obtained with 10 ng ml^{-1} $VEGF_{165}$. On a molar basis, $VEGF_{165}$ was 10-fold more potent than $VEGF_{20}$, demonstrating for the first time that a VEGF peptide fragment acts as a partial agonist. Specific activation of NOS was confirmed by the dose-dependent attenuation of $VEGF_{165}$- and $VEGF_{20}$-stimulated NO release by N^G-monomethyl-L-arginine with IC_{50} values of 3.4 μM and 7.0 μM, respectively (Fig. 2C). The inactive isomer D-NNA had no effect on VEGF-induced NO release (data not shown). Neural and endothelial isoforms of cNOS are dependent on calcium for their activation (Lowenstein and Snyder, 1992).

Our data suggested, therefore, that VEGF-evoked NOS activity is Ca^{2+}-dependent because the removal

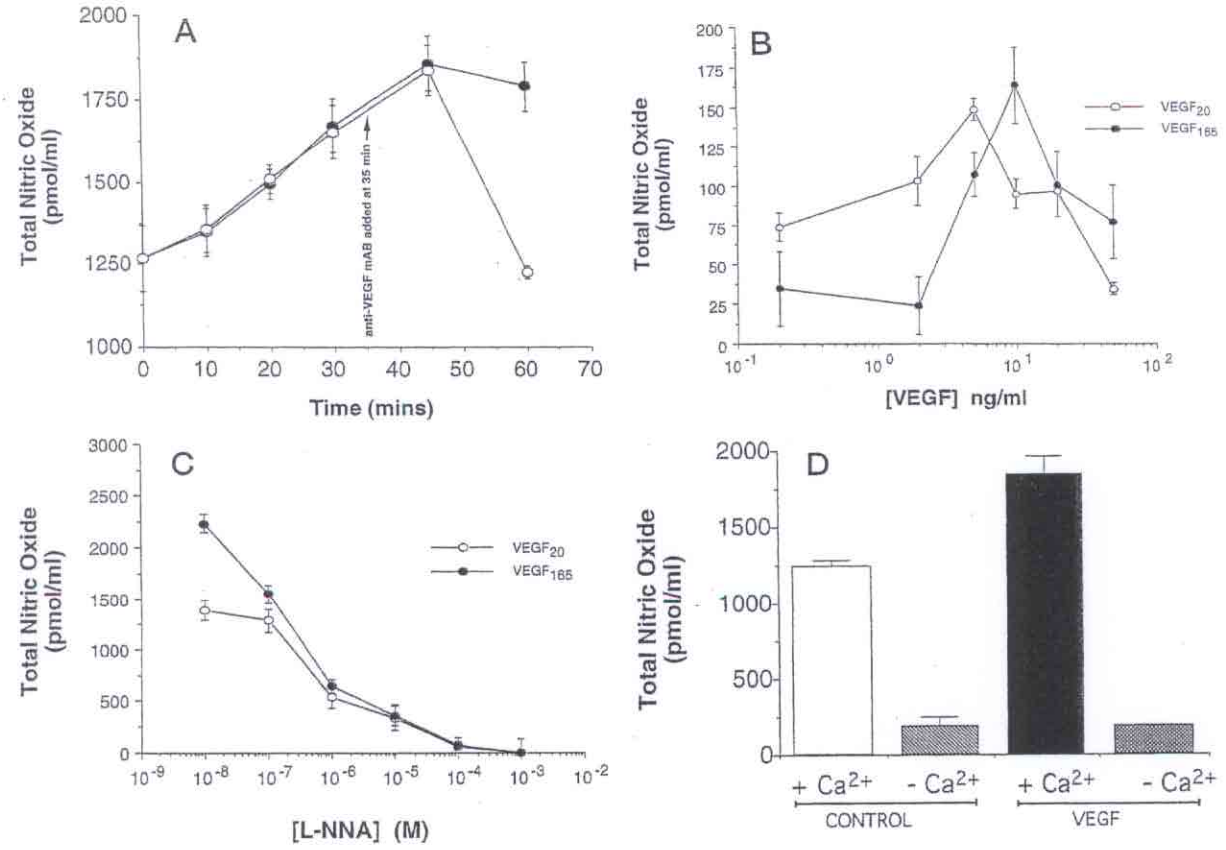

Figure 2.

VEGF activates the endothelial constitutive NO synthase in trophoblast cells. A, Time-dependent NO release in response to 10 ng/ml $VEGF_{165}$ (*solid circle*) or in the presence of 50 ng/ml anti-VEGF neutralizing mAb added after 35 minutes of stimulation with 10 ng/ml $VEGF_{165}$ (*open circle*). B, Dose-dependent release of NO in response to $VEGF_{165}$ (*solid circle*) and $VEGF_{20}$ (which is composed of the first 20 N-terminal amino acids of VEGF) (*open circle*). C, Effect of inhibition of NO synthesis by N^G-monomethyl-L-arginine (L-NNA) on $VEGF_{165}$ (*solid circle*) and $VEGF_{20}$ (*open circle*) -induced NO release. D-NNA has no inhibitory effect on VEGF-induced NO release. D, VEGF-mediated NO release in normal extracellular Ca^{2+} (*solid column*) and low Ca^{2+} buffer (*cross column*; 150 nM external Ca^{2+}) on VEGF-mediated NO release. Basal release of NO under normal extracellular Ca^{2+} (*open column*) and low Ca^{2+} buffer (*hatched column*). Cells were stimulated for a maximum period of 30 minutes (or as indicated) with the agonist in the presence or absence of the above agents. The reaction was terminated by the removal of supernatant. The supernatant was immediately stored at −80°C for NO analysis using an NO chemiluminescence analyzer. All results shown have been corrected for background levels of NO present in the media and, in the case of the dose-response experiments, for the background levels of NO present in the media, and medium plus cells were subtracted from the VEGF-mediated values. Data are expressed in pmoles of NO per milliliter. The results are expressed as mean ± SEM of a typical experiment (quadruplicate determinations per experiment of three similar experiments).

of extracellular calcium completely abolished the ability of VEGF to stimulate NO release ($p < 0.001$, $n = 2$; Fig. 2D). Moreover, the autocrine activation of NO biosynthesis as basal NO in the presence of calcium was significantly higher than that following the removal of extracellular calcium ($p < 0.001$, $n = 2$; Fig. 2D). This finding suggested the presence of one of the cNOS isoforms in trophoblasts, and RT-PCR—with primers directed against endothelial cNOS—confirmed that the endothelial isoform of the cNOS enzyme was expressed in trophoblast cells. (Fig. 1A).

The NO response to $VEGF_{165}$ is specifically blocked by the addition of anti-VEGF mAb (Kim et al, 1992) by 94.51% ± 4.49% ($p < 0.001$, $n = 3$; Fig. 3A), and the

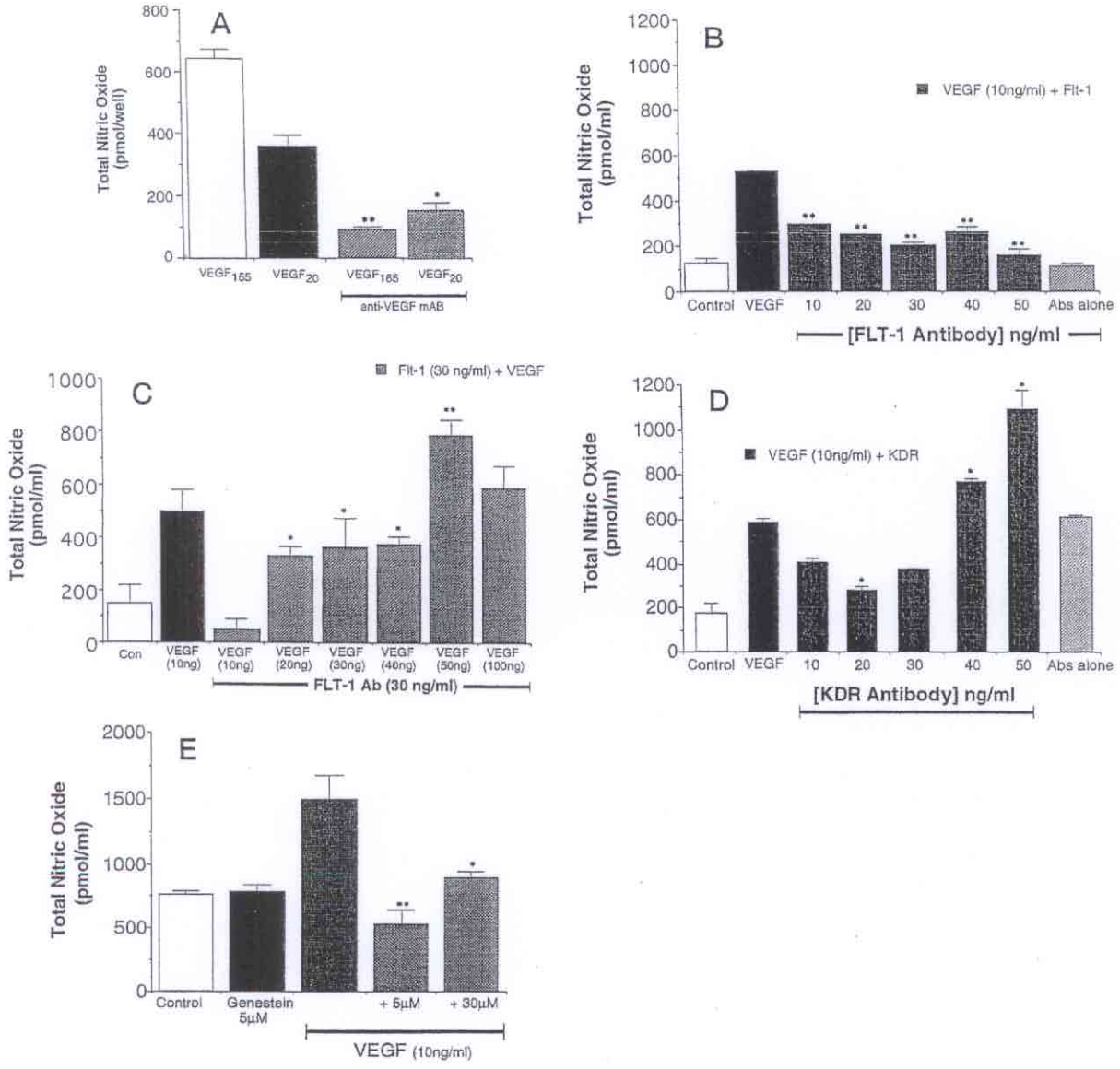

Figure 3.

VEGF stimulates NO release by the activation of the Flt-1 receptor in trophoblast cells. A, Inhibition of $VEGF_{165}$- and $VEGF_{20}$-stimulated NO release by addition of a neutralizing anti-VEGF mAb. The cells were incubated with either 10 ng/ml $VEGF_{165}$ (*open column*) or 5 ng/ml $VEGF_{20}$ (*solid column*) alone or in the presence of an anti-VEGF mAb at 50 ng/ml (*hatched column*) or 25 ng/ml (*crossed column*) for $VEGF_{165}$ and $VEGF_{20}$, respectively. B, Inhibition of $VEGF_{165}$-stimulated NO release by increasing concentrations of a polyclonal anti–Flt-1 antibody. $VEGF_{165}$ alone (10 ng/ml) (*solid column*) stimulates NO release above basal value (*open column*), and increasing concentrations of a polyclonal anti–Flt-1 antibody (*shaded column*) inhibited NO release, whereas anti–Flt-1 antibody alone at 40 ng/ml (*hatched column*) had no effect. C, Displacement of 30 ng/ml anti–Flt-1 antibody by increasing concentrations of $VEGF_{165}$. D, Effect of increasing concentrations of a polyclonal anti-KDR antibody on $VEGF_{165}$-stimulated NO release. E, Effect of 10 ng/ml $VEGF_{165}$ on NO release in cells preincubated for 60 minutes with genistein at 5 μM (*hatched column*) and 30 μM (*crossed column*) before the addition of $VEGF_{165}$. In these experiments, cells were incubated with $VEGF_{165}$ (unless otherwise stated) in the presence or absence of the anti-VEGF, anti–VEGF-receptor–neutralizing antibodies, or genistein for 2 hours at 37°C, and the conditioned media were collected for NO analysis. The results are expressed as mean ± SEM from three independent experiments performed in quadruplicate determinations per experiment. All results shown have been corrected for background levels of NO present in the medium alone. Data are expressed in pmoles of NO per milliliter. $^{*}p < 0.001$, $^{**}p < 0.0001$ compared with 10 ng/ml VEGF-stimulated NO release in the absence of any other test agent using unpaired Student's *t* test. In C, the *p* values represent a significant release of NO caused by increasing concentrations of VEGF as compared with 10 ng/ml VEGF-evoked NO release in the presence of 30 ng/ml Flt-1 antibody.

addition of an anti–Flt-1 receptor antibody inhibited NO release in a dose-dependent manner ($p < 0.001$, $n = 3$; Fig. 3B), an effect which was shown to be reversible with higher concentrations of $VEGF_{165}$ (Fig. 3C), indicating that VEGF-stimulated NO release is mediated via the Flt-1 receptor in trophoblast cells. This is the first demonstration of the ability of Flt-1 receptor to stimulate NO release in response to VEGF. The only other biologic function attributed to the Flt-1 receptor is the ability of peripheral blood monocytes (which express Flt-1 and not KDR receptors) to migrate in response to VEGF (Clauss et al, 1996). Although the present study also showed that the addition of an anti-KDR receptor antibody at 20 ng/ml partially inhibited NO release, at high concentrations (40 to 50 ng/ml), it significantly increased VEGF-mediated NO release ($p < 0.01$, $n = 3$). This may be due to displacement of VEGF from the KDR receptor by this antibody, which would cause an increase in VEGF levels, thereby further activating the Flt-1 receptor. As these receptors have tyrosine kinase activity and as genistein is a putative inhibitor of $pp60c^{-src}$ kinase activity, genistein might be expected to inhibit VEGF-stimulated NO release. Figure 3E shows that genistein completely inhibited VEGF-stimulated NO release (5 μM genestein; $p < 0.001$, $n = 3$). Further studies have shown that the action of genistein is due to the specific inhibition of VEGF-induced phosphorylation of the Flt-1 receptor. Preincubation of confluent trophoblasts with genistein (30 μM) or lavadustin A (26 μM) for 30 minutes followed by the addition of VEGF (10 ng/ml) in the presence of each inhibitor for 2 hours resulted in a decrease in intensity of the 2 major phosphorylated bands of the Flt-1 receptor at 140 kd (laser densitometry (LD) 623.9, 50% decrease; LD 455.4, 63% decrease) and 145 kd (LD 193.7, 64% decrease; LD 141.2, 74% decrease), respectively, compared with 10 ng/ml VEGF alone (LD 1227.5, 534.1) (Fig. 4A). In contrast, the KDR receptor underwent minimal phosphorylation, and no major bands of 140 kd were detected. Bands detected at 50 and 55 kd showed no increase in response to 10 ng/ml VEGF, and the inhibitors also had no effect on the observed phosphorylation (Fig. 4B). These results provide conclusive evidence that VEGF mediates NO release through activation and phosphorylation of the Flt-1 receptor.

As VEGF is implicated in angiogenesis associated with tumor growth (Clauss et al, 1996) where VEGF is induced by hypoxia in glioblastoma cells (Shweiki et al, 1992), we postulated that VEGF may play a role in vasodilatation of blood vessels by stimulating NO release. To examine this possibility, we exposed confluent monolayers of HUVE cells to $VEGF_{165}$ at 1 and 10 ng/ml, which caused release of NO into the culture medium that was blocked by anti–Flt-1 receptor antibody (Fig. 5). The presence of Flt-1 receptors on nonproliferating adult endothelium (Jakeman et al, 1992) together with the ability of Flt-1 receptor to stimulate NO release suggests a role for Flt-1 receptor

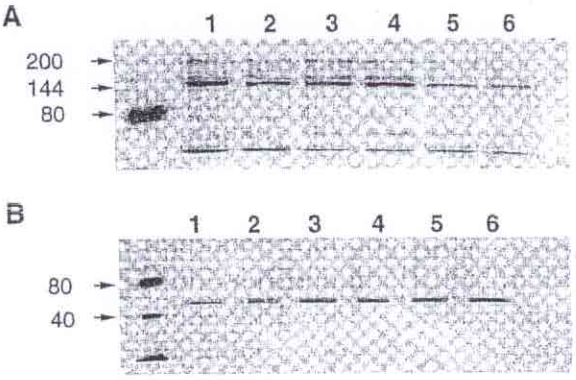

Figure 4.
Effect of genistein and lavadustin A on phosphorylation of the Flt-1 and KDR receptors by VEGF. Cells were incubated with serum free media (Lane 1), 30 μM genistein (Lane 2), 26 μM lavadustin A (Lane 3), and either 10 ng/ml VEGF alone (Lane 4) or in the presence of genistein (Lane 5) and lavadustin (Lane 6) for 2 hours at 37°C. Cell extracts were obtained in 0.5 ml of immunoprecipitation buffer and incubated with either 10 μg/ml anti-flt or anti-KDR antibody followed by SDS-PAGE and incubation with PY20 antiphosphotyrosine antibody. A, Inhibition of VEGF-induced phosphorylation of the flt-1 receptor by genistein and lavadustin A. Laser densitometry (LD) analysis of the two major bands at 140 kd and 145 kd shows that flt-1 undergoes autophosphorylation that is not attenuated by the addition of the inhibitors alone ($LD_{con140kd}$ = 940.6; $LD_{con145kd}$ = 455; LD_{gen} = 937.8; LD_{gen} = 412.9; LD_{lav} = 933.9; LD_{lav} = 457.3). In contrast, 10 ng/ml VEGF caused an increase in tyrosine phosphorylation (LD_{VEGF} = 1227.5; LD_{VEGF} = 534.1), which was inhibited by both genistein by 50% ($LD_{gen+VEGF}$ = 623.9; $LD_{gen+VEGF}$ = 193.7) and lavadustin A by 63% ($LD_{lav+VEGF}$ = 455.4; $LD_{lav+VEGF}$ = 141.2). B, KDR undergoes minimal phosphorylation and shows no change in response to VEGF, genistein, or lavadustin A. No major bands of the expected size (140 kd) were detected, and the 2 bands detected (at 50 and 55 kd) show no difference between unstimulated cells and cells incubated with 10 ng/ml VEGF alone or in the presence of the inhibitors.

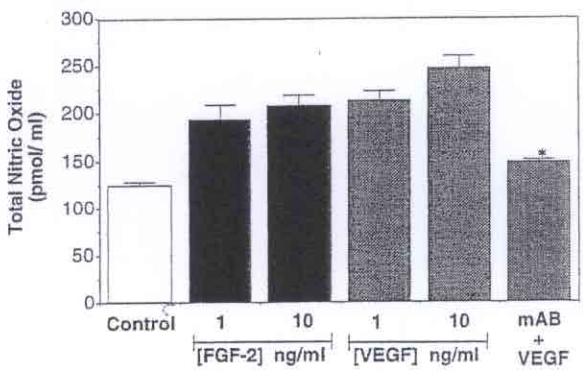

Figure 5.
VEGF stimulates NO release in cultured human umbilical vascular endothelial cells. Addition of FGF-2 (*solid column*) or $VEGF_{165}$ (*hatched column*) at 1 and 10 ng/ml caused an increase in NO release into the culture medium above the basal level (*open column*). Using 5-fold more of anti-VEGF mAb (*crossed column*), 10 ng/ml $VEGF_{165}$-stimulated NO release is significantly inhibited ($^*p < 0.01$).

in the maintenance of endothelial cell integrity and vessel patency. Although not eluded to by the authors, recent in vivo studies on the effects of VEGF in inducing the development of brush-like vessels in the precapillary region on chorioallantoic membrane of chicken embryos showed an increased vessel diameter (Birkenhager et al, 1996).

Whereas high levels of NO are cytotoxic (Hibbs et al, 1988), lower levels of NO may inhibit growth of mesangial cells (Appel, 1990) and vascular smooth muscle (Garg and Hassid, 1989) without toxic effects. This latter action on NO may, however, vary among cell types (Firnhaber and Murhy, 1993). The addition of increasing concentrations of sodium nitroprusside (SNP) to trophoblasts caused an increased in DNA synthesis at picomolar concentrations, whereas at nano- and micromolar concentrations, SNP produced marked inhibition in [^{3}H]thymidine incorporation (Fig. 6). Although VEGF at 1 to 2 ng/ml is a weak mitogen for trophoblast cells (Charnock-Jones et al, 1994), VEGF at 10 ng/ml, when it generates maximal NO production, has no mitogenic activity (Table 1). More important, endogenous basal trophoblast proliferation, as measured by DNA synthesis, was markedly potentiated in the presence of anti–flt-1 antibody, whereas DNA synthesis remained at basal levels when the KDR receptor was blocked by anti-KDR antibody (Fig. 7). The increased DNA synthesis in the presence of anti–flt-1 antibody may then be due to inhibition of NO production. It is proposed that over production of trophoblast-derived NO via the Flt-1 receptor may negatively regulate DNA synthesis in these cells. Intrauterine growth restriction is characterized not only by the abnormal Doppler wave forms in the umbilical artery, but also by the relatively small-sized placenta

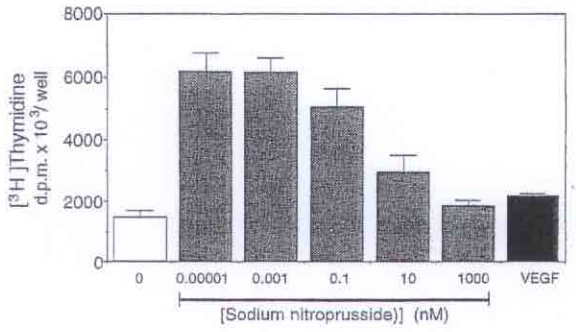

Figure 6.
Effect of increasing concentrations of sodium nitroprusside (SNP) on DNA synthesis in human trophoblast cells. Increasing concentrations of SNP [10^{-14} M to 10^{-6} M] (*hatched columns*) caused a dose-dependent attenuation in [^{3}H]thymidine incorporation. VEGF at 10 ng/ml (*solid columns*) or control (*open column*) had no effect on DNA synthesis. The results are expressed as mean ± SEM from three independent experiments performed in triplicate determinations per experiment. Cell viability was assessed by trypan blue exclusion at the end of each experiment.

Table 1. Effect of VEGF and EGF on DNA Synthesis

Stimulus	[^{3}H]Thymidine (dpm/well)
Control	2357 ± 379.2
VEGF	2652 ± 245.8^{ns}
EGF	4819 ± 653.4*

Quiescent cells were incubated with [^{3}H]-thymidine in the presence of either vehicle (control, $n = 10$), VEGF (10 ng/ml) ($n = 7$) or EGF (2 ng/ml) ($n = 4$) for 24 hours. DNA synthesis was determined as described in "Materials and Methods." VEGF did not stimulate DNA synthesis in these trophoblast cells, whereas EGF stimulated [^{3}H]-thymidine incorporation.

Data are expressed as mean ± SEM of independent experiments performed in triplicate determinations per experiment. Statistical analysis was performed using Student unpaired t test.

* $p < 0.01$ when compared with control values.

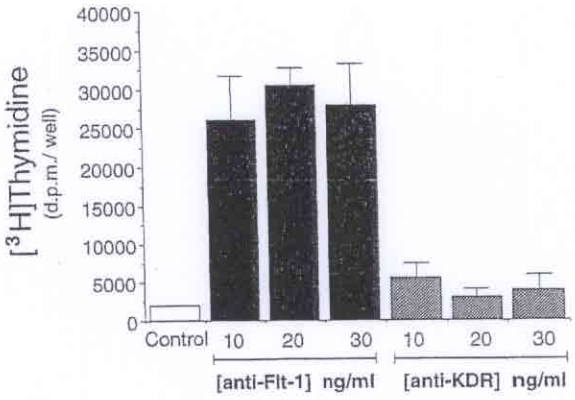

Figure 7.
Effect of selective blockade of the VEGF receptor subtypes on endogenous DNA synthesis in human trophoblast cells. Cells were incubated with anti–Flt-1 (*solid*) or anti-KDR (*hatched*) antibodies at increasing concentrations of 10 to 30 ng/ml for 24 hours. The addition of the anti–Flt-1 antibody caused an increase in endogenous DNA synthesis above basal levels (*open column*), whereas the addition of anti-KDR antibody showed no increase above basal growth. Cells were washed and lysed as described in "Materials and Methods," and data are expressed as a mean ± SEM of three independent experiments performed in triplicate determinations per experiment. Cell viability was assessed by trypan blue exclusion at the end of each experiment.

and depleted numbers of arteries and arterioles in the tertiary stem villi (Jackson et al, 1995). Rutherford et al (1995) recently reported that NOS activity was elevated in placenta from intrauterine growth-restricted (IUGR) pregnancies, and the current study also shows that the intensity of localization of immunoreactive endothelial cNOS was markedly increased in IUGR ($n = 5$) compared with normal ($n = 6$) placentae (Fig. 8). These results support our proposition that excess trophoblast-derived NO may limit the growth of the placenta and that one of the functions of Flt-1 is to suppress VEGF-induced proliferation mediated via the KDR receptor, in endothelial cells as well as in trophoblast cells.

The expression of the Flt-1 receptor on the invasive extravillous trophoblast cells (Charnock-Jones et al, 1994) and the localization of VEGF in the maternal decidua (Ahmed et al, 1995; Sharkey et al, 1994) has

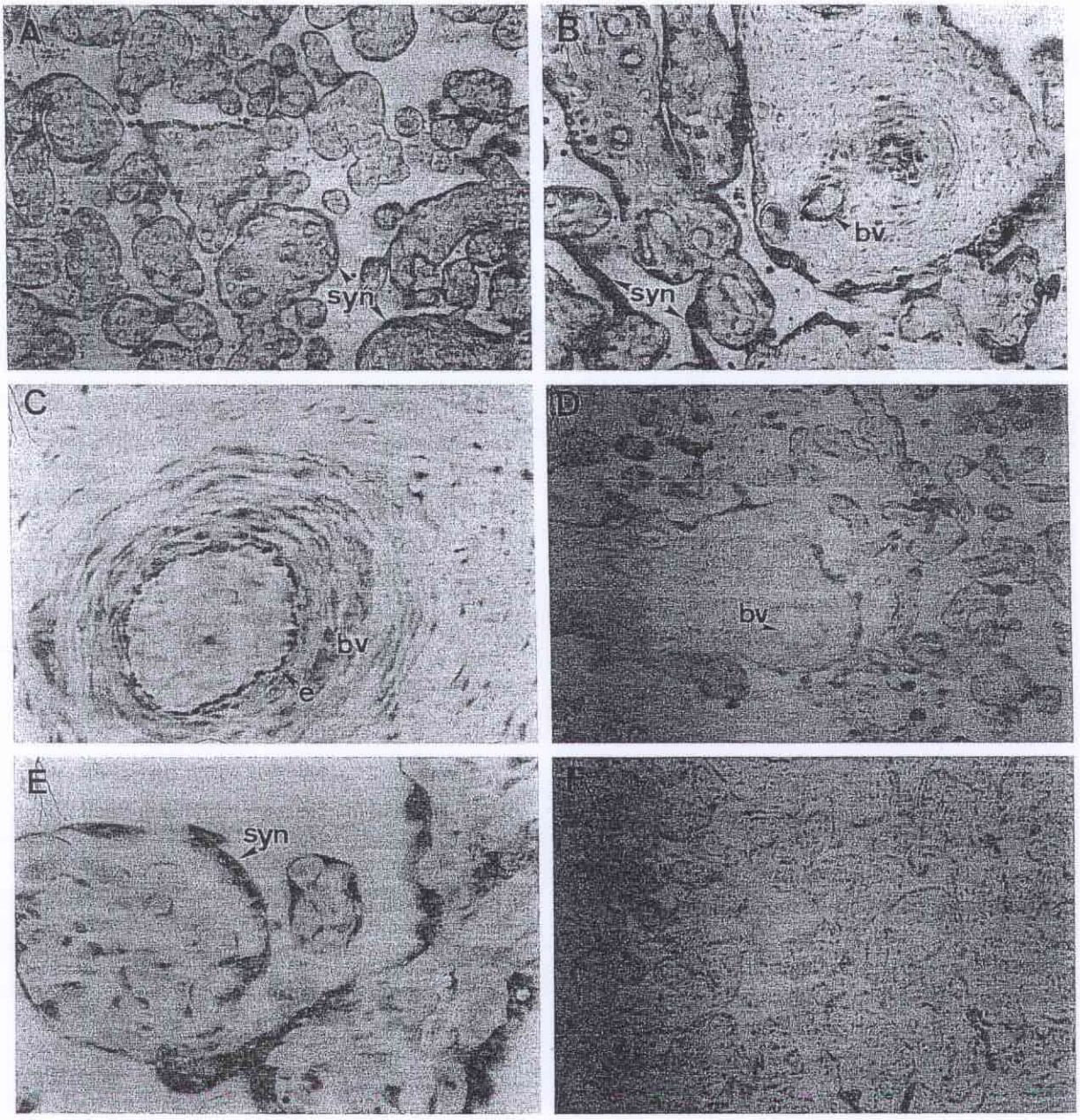

Figure 8.

Immunolocalization of endothelial cNOS in placental villi of growth-restricted and normal pregnancy. Immunohistochemistry was performed on placenta from intrauterine growth-restricted (IUGR) ($n = 5$) and gestationally matched normal ($n = 6$) pregnancies. A to C, Placental sections from severely growth-restricted pregnancies showing strong positive immunostaining for endothelial cNOS in the syncytium (*Syn*) corresponding with the fused syncytiotrophoblast/cytotrophoblast lining of the villi. There was also intense immunoreactivity around fetal blood vessels (*bv*) and in the endothelial cells (*e*) lining the vasculature and the cells within the mesenchymal stromal core of the villous tissue. D and E, Placental sections from uncomplicated normal pregnancies. Endothelial cNOS immunoreactivity was sparsely localized in the syncytium (*Syn*) of the placental villi of normal pregnancies. These was no detectable immunostaining in the mesenchymal stroma or around the fetal blood vessels. F, Control in which the primary anti-cNOS antibody was substituted with nonimmune goat antisera. Original magnifications: ×280 (A); ×560 (B, C, and E); ×140 (D and F).

suggested that VEGF may act as a chemoattractant for the invading trophoblast cells. A recent study in the guinea pig showed that increased dilation of the uteroplacental arteries due to NO release was a prerequisite for trophoblast invasion and remodeling of the endothelium (Nanaev et al, 1995). It is interesting to note that NOS activity is highest during the first trimester (Rutherford et al, 1995), during which time the placental VEGF level is also maximal (Khaliq et al, 1996a). Moreover, VEGF does not stimulate the migration of trophoblasts in a transwell migration chamber system (unpublished data). Based on the current findings, we propose that VEGF is not a chemoattractant for invading trophoblast, but that it functions, instead, as an aid to the penetration of invading extravillous trophoblasts into the maternal spiral arte-

rioles through its ability to stimulate NO. Hence, we hypothesize that: (a) the general ability of trophoblasts to remodel the endothelium by penetrating the spiral arterioles may correspond with the ability of local autocoids, such as VEGF, to stimulate NO; and (b) the failure to generate adequate amounts of trophoblast-derived NO may predispose women to pre-eclampsia.

A role for VEGF-mediated NO release via the Flt-1 receptor in the development and maintenance of the utero-placental unit is further supported by the current findings. Development of pregnancy-induced hypertension is associated with impaired blood flow to the placenta and coagulopathy leading to severe maternal hypertension and fetoplacental central compromise. As VEGF (Ahmed et al, 1995; Sharkey et al, 1994), Flt-1 (Ahmed et al, 1995; Charnock-Jones et al, 1994), and endothelial cNOS (Myatt et al, 1993; Rutherford et al, 1995) are all localized to the syncytium, the layer of trophoblast cells that line the intervillous space occupying the analogous position of the endothelium in a blood vessel, we propose that the vasodilatory and anti-platelet aggregatory properties of NO generated after Flt-1 activation by the villous trophoblast cells may serve to regulate the hemodynamics of the maternal-fetal interface. Decrease in maternal endothelial cNOS activity, as seen in pregnancy-induced hypertension, may be a result of reduced expression of VEGF or its Flt-1 receptor. This study conclusively shows that human trophoblast cells contain the high-affinity Flt-1 receptor, which is functionally coupled to cNOS activation and NO release, and that VEGF acts as an autocrine regulator of NO biosynthesis within the trophoblasts to limit DNA synthesis and probably controls placental growth.

Materials and Methods

Patient Selection and Tissue Collection

Human placenta was obtained from full-term uncomplicated pregnancies and gestationally-matched tissue from pregnancies complicated by severe asymmetrical IUGR delivered by Cesarean section. IUGR placental tissues were obtained from women with absent-end diastolic flow velocity and small-for-date babies (fetal weight less than 5th centile for gestational age) (Firnhaber and Murrhy, 1993). Placental tissues were taken from a central location lying between the basal and chorionic plates, washed thoroughly in phosphate buffered saline, and immersed in 10% formaldehyde immediately after collection. Ethical committee approval for tissue collection was obtained from the South Birmingham Ethical Committee.

RT-PCR

Total cellular RNA was extracted from first-trimester trophoblast cells generated by repeated passaging of trophoblast cells obtained from first-trimester chorionic villi by chorionic villous sampling. This cell line provides a convenient model for the behavior of primary cytotrophoblast (Diss et al, 1992) because the cells exhibit most of the characteristics of first-trimester cytotrophoblast: they are cytokeratin-positive and CD68- and CD45-negative, secrete human chorionic gonadotrophin and parathyroid hormone-related protein (Ahmed et al, 1995), and show mitogenic response to epidermal growth factor. Total cellular RNA was also extracted from term placenta obtained at the time of elective cesarean section, and 1 μg was reversed transcribed with MoMLV reverse transcriptase to synthesis cDNA. The specific oligonucleotide primers then used for amplification of cDNA by PCR were as follows: (a) iNOS primers: sense, 5′-AAGCCCCACAGTGAAAGAACAT-3′; antisense, 5′-ATGTACCAAGCCATTGAAGGGG-3′; (b) eNOS primers: sense, 5′-CCCCCCGA GCTCCCCGCTAAC-3′; antisense, 5′-GCAGTCCCGGGCATCGAACAC-3′; (c) flt-1 primers: sense, 5′-GTGGAAGAAATGGCAAACAA-3′; antisense, 5′-ACAG GTCAGAAGCCCTATTT-3′; (d) KDR primers: sense, 5′-GAAATGACACTGGAGC CTAC-3′; antisense, 5′-TTCCCAGTTGAAGTCAATCC-3′; (e) GADPH primers: sense, 5′-CTACTGGCGCTGCCAA-GGCTGT-3′; antisense, 5′-GCCATGAGGTCCA CCA-CCCTGT-3′. The predicted iNOS PCR product size was 1197 nt, whereas that of the endothelial cNOS product was 424 nt. The flt-1 and KDR PCR product sizes were 380 nt and 500 nt, respectively. Finally, the GAPDH housekeeping gene PCR product was 358 nt.

Immunocytochemistry

Immunocytochemistry was performed as described previously (Ahmed et al, 1995). Cells were fixed in suspension in a 4% formaldehyde solution and then spun down at 500 rpm for 5 minutes onto a glass slide. The slides were left overnight and processed the next day. Endogenous peroxidase activity was quenched by incubating the slides in methanol with the addition of 0.3% (v/v) of hydrogen peroxide for 10 minutes. Primary antibody, anti-VEGF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, California) is a rabbit polyclonal antibody raised against a 20 amino acid synthetic peptide corresponding with residues 1 to 20 mapping at the amino terminus of human VEGF (Plate et al, 1992), which is highly specific but cross-reacts with mouse and rat VEGF. Amplification of the primary antibody reaction was achieved using a goat anti-

rabbit secondary antibody for 30 minutes followed by a complex of streptavidin (DAKO, Ltd., Bucks, United Kingdom) and biotinylated peroxidase (DAKO, Ltd.) for a further 30 minutes. Finally, the binding was visualized by incubating the sections with 0.5 mg/ml diaminobenzidine (Sigma Chemical, Ltd., Poole, Dorset, United Kingdom) and 0.01% hydrogen peroxide in phosphate buffer saline (PBS) for 5 minutes to form the insoluble antigen-antibody complex. Sections were then counter-stained with Mayer's hematoxylin, dehydrated, and mounted. To test the specificity of the immunocytochemical staining, the primary antibody was replaced with goat nonimmune serum. Secondary and anti-human mouse monoclonal cytokeratin were commercially obtained from DAKO, Ltd.

Immunohistochemistry was also performed to localize endothelial cNOS on the normal term ($n = 6$) and IUGR placentae ($n = 5$). Serial 4-μm sections of formalin-fixed, paraffin-embedded tissue were used. Primary antibody, anti-endothelial cNOS antibody (Affiniti Research Product, Ltd., Exeter, United Kingdom) is an affinity-purified rabbit polyclonal antibody raised against a 20.4-kd protein fragment corresponding with amino acids 1030 to 1209 of human endothelial cNOS.

Preparation and Stimulation of Cells

The first-trimester trophoblast cells were maintained in 175-cm^2 flasks in a 1:1 mixture of DMEM Hams F12 nutrient mixture (DMEM/F12) containing 15% (v/v) fetal calf serum (FCS), 1% l-glutamine, and 1% antibiotic mixture (5000 IU/ml penicillin and 5000 μg/ml streptomycin) at 37°C in 95% O_2 and 5% CO_2 at 95% humidity. The culture medium was changed after 48 to 72 hours; confluent monolayers were trypsinized with EDTA-trypsin and plated into multiwell culture plates at 150×10^3 cells/well in 24-well plates. When monolayers attained over 80% confluency, the medium was replaced with serum-free DMEM/F12 for 24 hours. Stimulations were initiated by the addition of $VEGF_{165}$ (Saxon Biochemical, Hannover, Germany) or VEGF peptide fragment ($VEGF_{20}$) in serum-free DMEM/F12 with 0.2% bovine serum albumin. Experiments for dose-dependency were performed with increasing concentrations of $VEGF_{165}$ (0.2 to 50 ng/ml), and the peptide fragment $VEGF_{20}$ and time-course experiments were initiated using 10 ng/ml $VEGF_{165}$ and 5 ng/ml $VEGF_{20}$. Cells were also pretreated for 30 minutes in low Ca^{2+} buffer (150 nM external Ca^{2+} in Hank's buffered saline) or with a tyrosine kinase inhibitor 5 μM or 30 μM genistein (Sigma Chemical, Ltd.). Stimulations were subsequently initiated by the addition of 10 ng/ml $VEGF_{165}$ for 30 minutes at 37°C. To show specific activation of NOS by VEGF, experiments were initiated in the presence of increasing concentrations of N^G-monomethyl-L-arginine (Sigma Chemical, Ltd.) and D-NNA (inactive isoform). To demonstrate that the NO release was mediated by VEGF and its Flt-1 receptor, anti-VEGF mAb and anti-flt polyclonal and anti-KDR polyclonal antibodies were added in increasing concentrations to confluent cells followed by the addition of 10 ng/ml VEGF (or as otherwise stated in the given figure legend). All experiments were performed in a final volume of 0.5 ml at 37°C and reactions were terminated by removal of the supernatant, which was immediately stored at −70°C for NO analysis.

The neutralizing anti-VEGF mAb was a gift from Dr. N. Ferrara (Genetech, South San Francisco, California), whereas the VEGF receptor neutralizing polyclonal antibodies were commissioned for the present study. Anti-flt antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding with amino acids 1312 to 1328 mapping at the carboxy terminus of the precursor form of the flt of human cell origin. It is highly specific for Flt-1, with no cross-reactivity with KDR, Flt-4, or other tyrosine kinase receptors, and can identify human forms of Flt-1. Anti-KDR antibody is an affinity-purified rabbit polyclonal antibody raised against a peptide corresponding with amino acids 1158 to 1345 mapping at the carboxy terminus of precursor form of the KDR/flk-1 of mouse cell origin. It is highly specific for KDR, with no cross-reactivity with other protein tyrosine kinase membrane receptors, and can identify human KDR.

Immunoprecipitation of Flt-1 and Visualization of Phosphotyrosine

Trophoblast cells were seeded in 80-cm^2 flasks and grown to 80% to 90% confluency; the medium was then replaced with serum-free DMEM/F12 for 24 hours. After preincubation with genistein (30 μM) or lavadustin A (26 μM) for 30 minutes, stimulation was initiated by the addition of VEGF 10 ng/ml for 2 hours at 37°C. Controls of unstimulated cells, genistein alone, lavadustin alone, and VEGF 10 ng/ml were also carried out. After incubations flasks were washed with ice-cold PBS, the protein was extracted by the addition of 0.5 ml immunoprecipitation buffer (50 mM Tris HCl (pH 7.4), 1% Igepal-CA630, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA (pH 7.4), 1 mM PMSF, 1 μg/ml aprotinin, leupeptin, pepstatin A, 1 mM Na_3VO_4, and 1 mM NaF). Lysates were precleared by the addition of 50 μl of protein A agarose bead slurry (10 minutes, 4°C), and protein concentrations were

determined by the BioRad protein assay. Lysates were diluted to 1 μg/μl in PBS and 5 μg of anti–flt-1 or anti-KDR antibodies added and incubated overnight at 4°C on an orbital shaker. The immunocomplex was captured by the addition of 100 μl of bead slurry followed by 2 hours of incubation (4°C, orbital shaker) and centrifugation (14,000 rpm, 5 seconds). The beads were then washed three times with immunoprecipitation buffer, resuspended in 50 μl of 2 × Lammil buffer, and boiled for 5 minutes to dissociate the immunocomplex from the beads. The beads were collected by a microfuge pulse and SDS-PAGE performed on 20 μl of each sample. Proteins were transferred to a nitrocellulose filter and blocked by incubation with 1% rabbit serum for 2 hours at room temperature in Tween Tris Buffered Saline (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20). Phosphorylated bands were visualized by incubation of the filter with PY20 antiphosphotyrosine mAb (1:1000) (Affiniti Research Products, Ltd., Exeter, United Kingdom) for 1 hour at room temperature. The nonradioactive ECL Western blotting system (Amersham International, Bucks, United Kingdom) was used to detect phosphorylated bands of the Flt-1 and KDR receptors.

Primary Culture of Umbilical Vein Endothelial Cells

Primary cultures of HUVE cells were isolated from fresh umbilical cords (Ahmed et al, 1994). The veins were cannulated and washed with 30 to 60 ml of PBS to remove blood clots. This was replaced by 10 ml of collagenase (1 mg/ml in PBS), incubated at room temperature for 10 minutes, and collected; veins were then rinsed with 10 ml of medium 199. Cells were centrifuged, resuspended in 5 ml of medium 199 with 15% FCS plus L-glutamine (1%) and antibiotics (5000 IU penicillin and 5000 μg/ml streptomycin), and plated in 25-cm^2 Falcon tissue culture flasks in an humidified 5% CO_2 atmosphere. The culture medium was changed after 48 to 72 hours confluent monolayers were trypsinized with EDTA-trypsin and plated into multiwell culture plates at 250 × 10^3 cells/well in 24-well plates. After stimulation with increasing concentration of $VEGF_{165}$ for 30 minutes, the culture medium was collected for assay by the NO chemiluminescence analyzer, as described below.

Measurement of NO

Levels of NO were measured in the gas phase using a Sievers NOA 270B Chemiluminescence Analyser (Sievers NOA, Boulder, Colorado). This system utilizes the reaction of NO with ozone to form activated nitrogen dioxide (NO_2*), which luminesces in the red and far-red spectrum. Samples of cell culture medium (100 μl) were injected into a nitrogen-purge vessel containing a 1% solution of sodium iodide in glacial acetic acid to liberate gaseous NO from dissolved NO and nitrite. The sample gas was then exposed to ozone in the reaction vessel to form activated nitrogen dioxide (NO_2*), which was detected by a red-sensitive photomultiplier tube; the output was recorded using an integrating pen recorder. For each sample, the area under the curve was converted to picomole NO using a calibration curve constructed after the analysis of a series of sodium nitrite standards.

Measurement of [^{3}H]-Thymidine Incorporation

DNA synthesis was assayed by measuring [^{3}H]-thymidine incorporation into DNA, as previously described (Ahmed et al, 1994). Cells were plated at a density of 20,000 cells/well in 24-well plates and grown in DMEM/F12 medium containing 10% FCS at 37°C in 95% O_2 and 5% CO_2 at 95% humidity. After 24 hours, the medium was replaced with DMEM/F12 medium containing 0.1% FCS DMEM/F12 for an additional 24 hours. The medium was replaced with fresh medium containing 0.1% FCS, [^{3}H]-thymidine (0.2 μCi/ml), and increasing concentrations of test agents (0.5 ml) and further incubated for 24 hours. The medium was then removed, and the cells washed with PBS and incubated with ice-cold 5% cold trichloroacetic acid for 5 minutes. Next, cells were washed with 100% ethanol before lysis by the addition of 0.5 ml of a solution containing PBS, 0.5% Triton, 200 mM NH_4OH, and 0.1% BSA. Lysed cells were added to 3 ml of scintillation fluid, and the radioactivity was determined by liquid scintillation counting in a β-scintillation analyzer. The results are expressed as mean ± SEM dpm/well from three independent experiments performed in triplicate determinations per experiment. Cell viability was assessed by trypan blue exclusion at the end of each experiment.

Statistical Analysis

All data are expressed as mean ± SEM, and statistical analysis was performed using Student's unpaired *t* test.

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