Pigment Epithelium-derived Factor Inhibits Angiogenesis via Regulated Intracellular Proteolysis of Vascular Endothelial Growth Factor Receptor 1*

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Pigment epithelium-derived factor (PEDF) has been identified as one of the most potent of endogenous negative regulators of blood vessel growth in the body. Here we report that PEDF is able to inhibit growth factor-induced angiogenesis in microvascular endothelial cells through a novel pathway requiring cleavage and intracellular translocation of the transmembrane domain of the VEGFR-1. Analysis of the subcellular distribution of PEDF in the presence or absence of PEDF. We are the first to demonstrate that the inhibitory effect of PEDF on VEGF-induced angiogenesis results from enhancing γ-secretase-dependent cleavage of the C terminus of VEGFR-1, which in turn inhibits VEGF-2-induced angiogenesis. In addition, PEDF was also able to regulate the phosphorylation of VEGFR-1, which itself can regulate VEGFR-2 signaling.

EXPERIMENTAL PROCEDURES

Reagents—Microvascular endothelial cell basal medium (MECBM) and growth supplement were from TCS Works (Buckingham, UK). Recombinant VEGF and γ-secretase activity kits were obtained from R & D Systems (Abingdon, UK). Recombinant PEDF was purchased from BioProducts MD (BioProducts MD LLC, MD). ProteoExtract™ subcellular proteome extraction kit was obtained from Merck. Neutralizing antibodies were initially a gift from Professor Asif Ahmed (Birmingham University, Birmingham, UK) and then obtained from Santa Cruz Biotechnology. All other materials were from Sigma unless otherwise stated.

Cell Culture—Bovine retinal microvascular endothelial cells (BRMECs) were isolated and cultured as described previously (16). Briefly, freshly isolated bovine retinas were homogenized by a Teflon-glass homogenizer. After trapping on an 83-μm nylon mesh, the microvessels were transferred into 2× modified Eagle’s medium containing enzyme mixture (500 μg/ml collagenase, 200 μg/ml Pronase, and DNase) at 37 °C for 20 min. The resultant vessel fragments were trapped on a 53-μm mesh, washed, pelleted, and plated out in microvascular endothelial cell basal medium (MECBM) with growth supple-

endothelial growth factor (VEGF) and fibroblast growth factor (4). This inhibitory effect was more profound than any of the other anti-angiogenic factors tested (i.e. angiotatin, thrombospondin-1, and endostatin). PEDF selectively inhibits the formation of new vessels from endothelial cells but does not appear to harm the existing vascular structure (6). The inhibitory effect of PEDF on vessel formation appears to be reversible when a transient and regulated angiogenesis occurs in situations including tissue repair after injury (7). A recent study by Ogata et al. (8) has provided clinical evidence to show that expression of VEGF and PEDF are inversely correlated during the development of retinal neovascularization, further supporting an anti-angiogenic role for PEDF in vivo.

Although numerous studies have shown that VEGF mediates endothelial cell behavior via VEGFR-2 (9–11), there is increasing evidence that VEGFR-1 plays a critical role in regulating VEGFR-2-mediated signaling (12–15). Based on these observations, we explored the possibility that PEDF regulates angiogenesis through VEGFR-1 using an in vitro angiogenesis model exposing microvascular endothelial cells to PEDF in the presence or absence of VEGF. We are the first to demonstrate that the inhibitory effect of PEDF on VEGF-induced angiogenesis results from enhancing γ-secretase-dependent cleavage of the C terminus of VEGFR-1, which in turn inhibits VEGF-2-induced angiogenesis. In addition, PEDF was also able to regulate the phosphorylation of VEGFR-1, which itself can regulate VEGFR-2 signaling.

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2 The abbreviations used are: PEDF, pigment epithelium-derived factor; BRMEC, bovine retinal microvascular endothelial cell; MECBM, microvascular endothelial cell basal medium; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor; PS, presenilin; TX-sol, Triton X-100-soluble; TX-insol, Triton X-100-insoluble; CTF, C-terminal fragment; EDANS, 5-(2-aminoethylamino)-naphthalene-1-sulfonic acid; 4-(4-dimethylaminophenylazo)benzoic acid.

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mament at 37 °C, 5% CO₂ for 3 days. The cells were used between passages 1 and 3.

Growth Factor Treatment of Quiescent Cultures—Nearly confluent cells were rendered quiescent for 45 min in serum-free MECBM before treatment with 100 ng/ml VEGF in the presence or absence of 100 ng/ml PEDF at 37 °C for indicated times. The cells exposed to medium alone acted as an unstimulated control group.

Time Course of PEDF Treatment—Near confluent BRMECs were treated with 100 ng/ml VEGF for 1 h at 37 °C followed by the addition of 100 ng/ml PEDF for 15 min, 30 min, 1 h, 12 h, 24 h, and 48 h. BRMECs were then subject to immunoprecipitation, Western blotting, and immunocytochemistry.

In Vitro Angiogenesis Assays—The angiogenic activity of endothelial cells was analyzed quantitatively using in vitro proliferation, migration, and tube formation models, as previously described (16, 17).

Proliferation—BRMECs were incubated in 96-well plates at 750 cells/well in MECBM with added growth supplement (TCS CellWork) for 24 h. The cells were transferred to serum-free MECBM for 45 min, at which time the medium was replaced with basal medium containing 1% fetal calf serum, and the various growth factors and the cells were incubated for 48 h. The relative cell number was determined by crystal violet staining monitored at 540 nm.

Tube Formation—Near confluent BRMECs were pretreated with growth factors for 48 h. The cells were detached and plated sparsely (2.5 × 10⁴/well) on 24-well plates coated with 12.5% Matrigel™ (Collaborative Research) and left overnight. The medium was then aspirated, and 250 µl/well of 12.5% Matrigel™ was overlaid on the cells for 2 h to allow polymerization of Matrigel™, followed by adding 500 µl/well of MCDB131 (a reduced serum supplement medium for the culture of microvascular endothelial cells) with 10% fetal calf serum for 24 h. The following day, the culture plates were observed under a phase contrast microscope and photographed at random in five different fields (×10). The tube length (mm/mm²) and number of capillary formations/microscope field were quantified.

Migration Assay (Wound Healing)—BRMECs incubated in MECBM with growth supplement in wells of a chamber slide were allowed to reach near confluence. To prevent cell proliferation at the wound edge, the cell monolayers were pretreated with 25 mg/ml 5-fluorouracil for 5 min followed by 48-h treatment with growth factors (in the presence of 5-fluorouracil). Then the cell monolayers were wounded by a 1-cm² pipette along one direction. Debris was removed, and fresh MECBM containing 1% fetal calf serum was added into the cultures at 37 °C for the time periods indicated. Cell migration was monitored and photographed at initial wounding and at time points indicated under a phase microscope (×4). The images were quantified using image analysis systems (NIH Image) and calculated as the distance (µm) migrated into the wound area.

Antisense Treatment—Antisenses (VEGFR-1, 5'-AAGCAGACACCCGAGCA-3'; VEGFR-2, 5'-GCTGCCTCTGATTTGTTGGG-3') and scrambled antisenses (VEGFR-1, 5'-ACTGTCACCTGACAGCCTTC-3'; VEGFR-2, 5'-TGTGTGGCATTGCGGTGTG-3') were transfected into BRMECs using the cationic liposomal carrier-DMRIE-C reagent (Invitrogen) that has previously shown efficacy without evidence of toxicity in endothelial cells (18). Consistent effects of the antisenses were produced when the antisense probes were added to the cultures every 24 h for 3 days. To determine an optimal concentration and specificity of antisense oligonucleotides, BRMECs were treated with different concentrations (2–10 µM) of the antisenses and scrambled antisenses followed by Western blotting analysis with rabbit polyclonal antibodies against the VEGFR-1 C-terminal domain or the VEGFR-2 C-terminal domain by using antibodies at a final concentration of 1:250 (Santa Cruz Biotechnology). The blots were stripped and reprobed with goat polyclonal anti-α-tubulin antibody (1:250) (Santa Cruz Biotechnology, Santa Cruz, CA). The band intensity was determined by LabWorks™ image acquisition and analysis software (Media Cybernetics).

Based on titration results the BRMECs were treated with 10 µM of the antisense and scrambled oligonucleotides over a 3-day period. The cultures were then exposed to growth factors for 48 h followed by assessment of tubule forming ability.

Western Blotting Analysis for Overall VEGFR-1 Expression—Details of the Western blotting procedure have been published previously (16). Whole cell lysate of BRMECs following treatment with VEGF and/or PEDF at 100 ng/ml for 48 h were subject to Western blotting. A rabbit polyclonal antibody against the VEGFR-1 C-terminal domain (Santa Cruz Biotechnology) was used at a final concentration of 1:250. The blots were stripped and reprobed with goat polyclonal anti-α-tubulin antibody (1:250) (Santa Cruz Biotechnology). The band intensity was determined by LabWorks™ image acquisition and analysis software (Media Cybernetics).

Cell Fractionation—Near confluent BRMECs were subjected to two extractions to obtain Triton X-1000-soluble (TX-sol) and -insoluble (TX-insol) fractions as previously described (17). The cells were first extracted with 500 µl of Triton lysis buffer (25 mM Tris, 150 mM NaCl, 0.25% sodium deoxycholate, 1 mM each of NaF, Na₃VO₄, EDTA, and phenylmethysulfonyl fluoride, 1 µg/ml of aprotinin, leupeptin, and pepstatin, 1% of Nonidet P-40, and Triton X-100) at 4 °C for 30 min. The cell lysates were centrifuged at 14,000 × g for 5 min, and the supernatant was collected as the TX-sol fraction. The remaining debris was further extracted with 500 µl of SDS buffer, which was the same as the Triton lysis buffer except that it contained 1% SDS instead of 1% Triton X-100. The extract buffer was vigorously pipetted before centrifugation at 14,000 × g for 5 min, and then the supernatant was collected as the TX-insol fraction. The proteins from the TX-sol and TX-insol fractions were resolved by SDS-PAGE followed by Western blotting analysis.

Neutralizing Antibodies—VEGFR-1 and VEGFR-2 were blocked by using neutralizing antibodies as described previously (16). In brief, BRMECs at near confluence were exposed to neutralizing antibodies to either VEGFR-1 or VEGFR-2 at 60 ng/ml for 1 h followed by the addition of VEGF, PEDF, or a combination thereof for 48 h. The cell lysates were then assessed by Western blotting to determine VEGFR-1 expression/translocation using rabbit polyclonal antibodies against the VEGFR-1 C-terminal domain or N-terminal domain (1:250).

γ-Secretase Activity Detection—The assay was carried out in a microplate reader using a γ-secretase Activity kit according to the manufacturer’s instructions. The principle of the assay is that the cell lysate is tested for secretase activity by the addition of a secretase-specific peptide conjugated to the reporter molecules (5-(2-aminoethyl)amino-naphthalene-1-sulfonic acid (EDANS) and 4-(4-dimethylaminophenylazo)benzoic acid (DABCYL)). In the uncleaved form the fluorescent emissions from EDANS are quenched by the physical proximity of the DABCYL moiety, which exhibits maximal absorption at the same wavelength (495 nm). Cleavage of the peptide by the secretase physically separates EDANS and DABCYL allowing for the release of a fluorescent signal. The level of secretase enzymatic activity in the cell lysate is proportional to the fluorometric reaction.

γ-Secretase Inhibition—Near confluent BRMECs in T25-cm² flasks were washed with phosphate-buffered saline and serum-free MECBM for 45 min. The cells were treated for 2 h with 2 ml/flask of MECBM containing 10% fetal calf serum and 13.5 µM γ-secretase inhibitor (Sigma). BRMECs were cultured with growth factors (VEGF or/and...
PEDF at 100 ng/ml for 48 h followed by either (a) assessment of tube forming ability or (b) Western blotting of lysates to determine VEGFR-1 distribution.

Reverse Transcription-PCR Analysis of Presenilin 1 Expression—Total RNA was isolated from BRMECs treated with growth factors by using TRIzol reagent (Invitrogen) and then reversed transcribed using Reverse-itTM (Abgene). Bovine presenilin 1 (PS1) transcripts were amplified using 5′-CCATGCCCTGGGCTTTTTT-3′ as forward primer and 5′-ATGCCTTGGAGTCTCAGT-3′ as reverse primer at 1.5 mM MgCl₂ and 54/93 °C annealing temperature. As control for the amount of mRNA input, we amplified bovine glyceraldehyde-3-phosphate dehydrogenase at 54/93 °C annealing temperature, 1.5 mM MgCl₂ concentration with forward primer 5′-GGGTCACTCATCTCTGCACCT-3′ and reverse primer 5′-GGTCATAAGTCCCTCCACGAG-3′. A total of 5-μl aliquots of amplified products were separated electrophoretically on a 1.2% agarose gel stained with ethidium bromide and illuminated with UV light and analyzed using NIH Image software.

Subcellular Protein Extraction—Purified proteins of four subcellular fractions from BRMECs treated with growth factors, including membrane, cytosolic, cytoskeletal, and nucleic fractions, were obtained using ProteoExtract™ subcellular proteome extraction kit. This kit preserves the integrity of the subcellular structures before and during extraction to prevent any mixing of the different subcellular compartments.

Immunoprecipitation and Western Blotting Analysis of Presenilin 1 Maturation—The membrane fractions obtained as above from BRMECs treated with growth factors over the indicated period times were immunoprecipitated with 10 μl of rabbit polyclonal antibody against the PS1 N-terminal domain (Santa Cruz Biotechnology) for 1.5 h at 4 °C followed by the addition of 20 μl of protein A/G-agarose overnight at 4 °C. The resultant pellet was subjected to SDS-PAGE and Western blotting by goat polyclonal antibody against the PS1 C-terminal domain (1:250) (Santa Cruz Biotechnology).

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**FIGURE 1.** The effect of VEGF and PEDF on in vitro angiogenesis. The effect of VEGF and PEDF, either alone or in combination on the proliferation (a), tubule formation (b), and migration (c and d) of microvascular endothelial cells. For proliferation studies preconfluent microvascular endothelial cells were treated with VEGF and/or PEDF for 48 h, and relative cell number was determined by crystal violet staining (a). For tubule formation, cells pretreated with VEGF and/or PEDF for 48 h were cultured between two layers of Matrigel™ in serum-deprived medium for 24 h. Morphometric quantification of the effect of VEGF and/or PEDF on in vitro tube formation was performed by image analysis (b). Migration was assessed using an in vitro wound healing model. To avoid cell proliferation at the wound edge the cell monolayers were treated with 25 mg/ml 5-fluorouracil for 5 min before treatment with growth factors for 48 h. The rate of wound closure was monitored. (c) shows the cultures at 24 h post wounding exposed to different growth factors. Scale bar, 400 μm. (d) depicts the distance of migration as a function of time. The data are shown as the means ± S.E. of three experiments. The asterisk denotes that PEDF significantly inhibits VEGF-induced migration (p < 0.05).

Immunochemistry—BRMECs grown to near confluent on glass coverslips were treated with growth factors over indicated time periods. After treatment, the monolayers were washed once with fully supplemented MECBM containing 5% fetal calf serum and then fixed with 4% paraformaldehyde for 10 min. The cells were permeabilized with 0.25% Triton X-100 for 5 min. After blocking by 0.1% bovine serum albumin, the cells were incubated for 1 h with primary antibodies against the PS1 C-terminus or Pan-cadherin (Santa Cruz Biotechnology) followed by an Alexa Fluor 488-conjugated or Alexa Fluor 594-conjugated secondary antibodies (Invitrogen). Hoechst 33342 (10 μg/ml) was added to visualize the nuclei. The cells were washed with phosphate-buffered saline, and the coverslips were mounted onto glass slides. The images were collected on a Leica confocal microscope using a Leica TCS NT “Image” program.

Tyrosine Phosphorylation Status of VEGFR-1—To determine the phosphorylation status of VEGFR-1 in whole cell lysate following treatment with VEGF and/or PEDF at 100 ng/ml, the phosphorylated proteins were immunoprecipitated from BRMEC lysate (200 μg/ml) by 10 μl of mouse anti-tyrosine phosphorylation monoclonal antibody (PY 20) for 1.5 h at 4 °C followed by the addition of 20 μl of protein A/G-agarose (Santa Cruz Biotechnology) overnight at 4 °C. The resultant pellet was subjected to SDS-PAGE and Western blotting by antibodies against VEGFR-1 as described above. Phosphorylation status in subcellular fractions was determined following immunoprecipitation with an anti-tyrosine phosphorylation antibody (PY 20) prior to SDS-PAGE and Western blotting by antibodies against VEGFR-1.

Statistical Analysis—All of the experiments were repeated at least three times. The results are expressed as the means ± S.E. Proliferation, migration, and tube formation was analyzed using a Student’s t test on log normalized data. The Mann-Whitney test was used to determine statistical significance of the laser densitometry data.
RESULTS

As expected exogenous VEGF stimulated microvascular endothelial cell proliferation, migration, and tubule formation by up to 80% as compared with the untreated cells ($p < 0.01$; Fig. 1). PEDF at 100 ng/ml resulted in a significant reduction of VEGF-induced endothelial cell proliferation and abolished migration and tubule formation ($p < 0.001$).

PEDF alone had no significant effect on migration or tubule formation when compared with the negative control (Fig. 1, b and c).

VEGFR-1 was shown to be a requirement for PEDF inhibition of VEGF-induced angiogenesis using an antisense oligonucleotide against VEGFR-1. Fig. 2a shows that VEGFR-1 antisense reduced VEGFR-1 expression in BRMECs in a concentration-dependent manner with an

**FIGURE 2.** The effect of antisenses on VEGF receptors expression. Both VEGFR-1 and VEGFR-2 antisense exhibited an inhibitory effect on expression of VEGFR-1 and VEGFR-2, respectively, in BRMECs in a dose-dependent manner with an 84% reduction of VEGFR-1 expression at 10 μM VEGFR-1 antisense oligonucleotide (a) or a 90% reduction of VEGFR-2 expression at 10 μM VEGFR-2 antisense oligonucleotide (c). Scrambled antisenses had no effect on VEGF receptor expression (b and d). Representative Western blot and densitometry analyses from at least three experiments are presented as the relative ratio of VEGFR-1 or VEGFR-2 to α-tubulin. The ratio relative to control is arbitrarily presented as 1. The error bars show the S.E. *, $p < 0.05$; **, $p < 0.01$ versus control. IB, immunoblot.
84% reduction of VEGFR-1 for 10 μM antisense without affecting VEGFR-2 expression. Scrambled VEGFR-1 antisense had no effect on VEGFR-1 expression (Fig. 2b). VEGFR-1 antisense abolished the inhibitory effect of PEDF on VEGF-induced tube formation but had no significant effect on VEGF-induced tube length (Fig. 3b). However, suppression of VEGFR-1 expression inhibited the VEGF-induced increase in capillary connections even in the absence of PEDF (Fig. 3b). Scrambled VEGFR-1 antisense had no effect on the inhibitory effect of PEDF. VEGFR-1 antisense also reversed the inhibitory effect of PEDF in the proliferation and migration assays (data not shown). These results clearly indicate that (a) PEDF inhibition of VEGF activity is highly dependent on VEGFR-1 in endothelial cells and (b) VEGFR-1 is able to regulate vascular maturation.

To determine whether VEGFR-2 is involved in the inhibitory effect of PEDF on VEGF-induced tube formation, VEGFR-2 antisense was used to suppress VEGFR-2 expression in BRMECs. In Fig. 2c, VEGFR-2 antisense exhibited a specific inhibitory effect on VEGFR-2 expression with a 90% reduction of VEGFR-2 at concentration of 10 μM without affecting VEGFR-1 expression, whereas scrambled VEGFR-2 antisense did not affect VEGFR-2 expression (Fig. 2d). VEGFR-2 antisense caused significant reduction of VEGF-induced tube formation (tube length and capillary connection) of BRMECs in the absence of growth factors (VEGF/or PEDF) (Fig. 3c).

To elucidate whether the involvement of VEGFR-1 in PEDF inhibitory activity is associated with a change in VEGFR-1 receptor density, whole cell lysates from BRMECs treated with VEGF and/or PEDF were subjected to SDS-PAGE and then probed by an antibody against VEGFR-1. VEGFR-1 was seen as a single band at the expected molecular mass of 180 kDa. Relative ratio of VEGFR-1 to α-tubulin showed no significant change under all conditions tested (Fig. 4a). To assess the subcellular distribution of VEGFR-1 following PEDF stimulation of endothelial cells, we extracted cells in 1% Triton X-100 and separated the TX-insol from the TX-insol fractions. Western blot analysis showed that full-length VEGFR-1 was found only in the insoluble fractions in negative controls, VEGF or PEDF treatment alone. PEDF in the presence of VEGF demonstrated the appearance of a significant amount of a
cleaved 80-kDa C-terminal domain of VEGFR-1 in the TX-sol fraction (Fig. 4b). PEDF did not appear to affect the extracellular N-terminal domain of VEGFR-1 in either the presence or absence of VEGF. These observations lead us to conclude that PEDF may induce cleavage of VEGFR-1 either within the transmembrane or the intracellular juxtamembrane region, resulting in a neutralizing antibody against VEGFR-1 added prior to VEGF/PEDF treatment abolished translocation of the C-terminal domain, whereas neutralization with VEGFR-2 reduced but did not completely block cleavage compared with the control (Fig. 4b). Furthermore, the finding that PEDF levels change in hypoxia-induced retinal neo-vascularization (19) allows us to predict that PEDF-induced cleavage of VEGFR-1 should occur at an early time. To test this hypothesis, the TX-insol fractions from BRMECs treated with PEDF over different times in the presence of VEGF were subject to Western blotting analysis of VEGFR-1 expression using antibody against the VEGFR-1 C-terminal domain. The data indicate that by 1 h after adding PEDF, a cleaved 80-kDa C-terminal domain of VEGFR-1 was present in the TX-sol fractions with a maximum level by 24 h post-treatment (Fig. 4c).

Intrigued by the observation that PEDF + VEGF induce a relocation of the intracellular domain of VEGFR-1 to the TX-sol fraction, we went on to test whether the possible cleavage of VEGFR-1 might be processed by γ-secretase. We first sought to examine whether γ-secretase activity was affected by PEDF. Pretreatment of endothelial cells for 48 h in the presence of PEDF resulted in a significant dose-dependent increase of γ-secretase activity in the total cell lysate: up to ~6-fold for PEDF treatment compared with VEGF treatment, which did not affect γ-secretase activity (Fig. 5a). Furthermore, combined PEDF and VEGF treatment did not increase γ-secretase activity above that achieved with PEDF alone. With the addition of a γ-secretase inhibitor, the inhibitory effect of PEDF on VEGF-induced tubule formation was completely abolished (Fig. 5b). Treatment of cells with γ-secretase inhibitor also blocked the translocation of the intracellular domain of VEGFR-1 to the TX-sol fractions induced by PEDF+VEGF (Fig. 5c). This indicates that PEDF appears to enhance γ-secretase activity leading to inhibition of angiogenesis, by way of cleaving VEGFR-1.

γ-Secretase is a complex consisting of four molecules. APH-1 and nicastrin form the initial complex, to which PS binds followed by PEN-2 binding. This complex is trafficked to the plasma membrane from the endoplasmic reticulum/Golgi in a mature functional form (20). Compelling evidence has emerged to support an essential role for PS in the enzymatic activity of γ-secretase (20). We analyzed whether an increase in γ-secretase activity induced by PEDF resulted from an elevation in PS expression. Reverse transcription-PCR was performed on RNA isolated from BRMECs treated with VEGF and/or PEDF using primers specific for bovine PS1. The expected size products of 188 bp were detected on the gel showing that ratio of PS1 mRNA relative to glyceraldehyde-3-phosphate dehydrogenase mRNA was not significantly changed under all conditions tested (Fig. 6a). However, PS endoproteolysis and the PS heterodimer represent activation of PS. In the membrane fractions of BRMECs, we show that after 30 min, PEDF results in an increase in the levels of both full-length and C-terminal fragment of PS1 with the greatest at 12, 24, and 48 h, compared with those of VEGF treatment only (Fig. 6b). To investigate changes in the subcellular localization of PS, triple immunostaining of PS1, a plasma membrane marker protein Pan-cadherin and a nuclear stain followed by confocal microscopy were performed. PS1 showed a predominant perinuclear staining under...
VEGF treatment only, whereas PEDF induced translocation of PS1 to the cell membrane (Fig. 6c).

We were then able to demonstrate the PEDF regulates the subcellular distribution of VEGFR-1 within endothelial cells. Immunoblots following subcellular fractionation for the N-terminal domain of VEGFR-1 in control cells and cells treated with VEGF, PEDF, and VEGF+PEDF revealed the presence of an 180-kDa VEGFR-1 in membrane, cytoskeletal, and nuclear fractions but not in the cytosolic fraction (Fig. 7a). Similarly staining for the C-terminal domain of VEGFR-1 detected an 80-kDa C-terminal domain of VEGFR-1 in the membrane, cytoskeletal, and nuclear fractions. An additional 80-kDa C-terminal domain of VEGFR-1 was observed in the cytosol of fractions treated with both VEGF and PEDF (Fig. 7a).

To assess the phosphorylation status of the VEGFR-1 receptor and its translocated domains, we immunoprecipitated phosphorylated proteins and, following Western blotting, immunolocalized with antibodies against VEGFR1. Consistent with a previous study by Itokawa et al. (21), VEGF induced an increase in autophosphorylation of VEGFR-1 compared with control with bands at 250 and 180 kDa in whole cell lysates (Fig. 7b). PEDF greatly reduced VEGFR-1 phosphorylation in both the 250- and 180-kDa bands, and this was greatest when PEDF was used in combination with VEGF (Fig. 7c). To test whether PEDF inhibited acute VEGFR-1 phosphorylation induced by VEGF, BRMECs were stimulated with 100 ng/ml VEGF for 1 h and then incubated with 100 ng/ml PEDF for different time periods. A significant reduction of both 180 and 250 kDa bands occurs after 1 h of PEDF addition (Fig. 7c).

Analysis of the subcellular fractions demonstrated that VEGF induced an increase in autophosphorylation of VEGFR-1 in membrane, cytoskeleton, and nuclear fractions. The antibody against the intracellular domain detected both the 180- and 250-kDa bands, whereas that against the extracellular domain of VEGFR-1 only detected the lower band. PEDF almost completely blocked the VEGF-induced tyrosine phosphorylation in all of the fractions regardless of whether the endothelial cells were cultured in the presence or absence of VEGF (Fig. 7c). Phosphorylation of the 80-kDa VEGFR-1 was not detected in the cytosolic domain.

**DISCUSSION**

This study provides a novel insight into the mechanisms by which PEDF via VEGFR-1 is able to inhibit VEGF-induced angiogenesis in microvascular endothelial cells. Two distinct signaling pathways are involved, (a) regulated translocation of the intracellular domain of VEGFR-1 and (b) VEGFR-1 phosphorylation. These observations further support a critical role for VEGFR-1 in the regulation of VEGFR-2.

The emergence of VEGFR-1 as a regulator of VEGFR-2-induced angiogenesis (12–15) led us to postulate that PEDF may inhibit neovascularization via VEGFR-1. The current study confirms this hypothesis because blockade of VEGFR-1 negates the inhibitory effect of PEDF on VEGF-induced angiogenesis. Furthermore, this requires the regulated intramembrane proteolysis of VEGFR-1. The cleavage of VEGFR-1 within or near its transmembrane domain and the release of the intracellular domain into the cytoplasm closely resemble that of γ-secretase-mediated processing of β-amyloid precursor (22, 23) and the Notch family (24, 25). We have confirmed through inhibition studies that γ-secretase is indeed the enzyme complex responsible for the cleavage of VEGFR-1 and have shown that if this cleavage is blocked, then PEDF is ineffective at inhibiting VEGF-induced angiogenesis. Functional studies have revealed that PS forms the major catalytic subunit of γ-secretase complex in conjunction with three other components (APH-1, PEN-2, and Nct) (20). Our studies reveal that the PEDF-induced increase in γ-secretase activity is not due to an increase in gene expression of PS but instead the trafficking of PS1 from its primary location at the perinuclear compartment to the plasma membrane. The most important and well studied post-translational modification of PS is proteolytic processing to generate an N-terminal fragment and C-terminal fragment (26). Endogenous functional PS1 in human brain tissue was found predominantly as the N-terminal fragment/C-terminal fragment (CTF) heterodimer (26). In support of this, our Western blotting
analysis indicated that PEDF induces an increase in not only full-length PS1 but also PS1-CTF in the membrane fractions, implying that PEDF enhances γ-secretase activity, at least partly via increasing PS levels and activation in the plasma membrane. An intriguing observation was that although PEDF alone was able to increase γ-secretase, regulated intramembrane proteolysis only occurred in the presence of VEGF. The presence of valine residues in the transmembrane domain of proteins serves as a substrate for γ-secretase (27), and sequence analysis has revealed that the amino acid sequence of the VEGFR-1 transmembrane domain is highly conserved and contains valine residues (28). This leads us to hypothesize that VEGF binding might cause a change of VEGFR-1 conformation, which in turn exposes the conserved valine residues to γ-secretase. Furthermore, γ-secretase cleavage of VEGFR-1 appears to differ from some of the other transmembrane receptors in that cleavage of the extracellular domain does not appear a prerequisite. For example, cleavage of the extracellular domain of Notch by tumor necrosis factor-α converting enzyme is required prior to transmembrane proteolysis and translocation of the intracellular domain (29).

It is possible that other angiogenic inhibitors may regulate endothelial cells via enhancing γ-secretase activity. For example, tumor necrosis factor α and interferon-γ, two inflammatory cytokines that inhibit growth factor-induced angiogenesis in vivo, were found to activate γ-secretase activity (30). Furthermore, co-expression of the four constituents of γ-secretase in cultured mammalian cells enhances γ-secretase activity (31). It is tempting to speculate that introduction of exogenous γ-secretase components into endothelial cells may lead to the cleavage of VEGFR-1 and block angiogenesis in the absence of PEDF. If true, it raises the question of whether γ-secretase would therefore constitute a better target for anti-angiogenesis therapies.

PEDF, in the presence of VEGF, elicits cleavage of VEGFR-1 with the resultant intracellular domain accumulating within the cytoplasm. This was surprising because γ-secretase cleavage of other receptors, i.e. Notch (24, 25), ErB-4 (32), and cadherin (33), results in translocation of the intracellular domain to the nucleus and binding to transcription factors. In contrast, PEDF elicited a decrease in VEGFR-1 nuclear localization, and this was coincident with inhibition of VEGF-induced angiogenesis by PEDF. Thus it seems reasonable to propose that PEDF acts as an inhibitor to VEGF-induced angiogenesis by disruption of VEGFR-1 and that its downstream signaling was due to cleavage of the intracellular VEGFR-1 domain and the reduction of its nuclear translocation. To confirm that the observed intracellular domain does not represent the production of a cross-reacting protein, the immunoreactivity of the VEGFR-1 C-terminal domain was also confirmed by a second antibody against an amino acid sequence from a different region of the VEGFR-1 C-terminal domain (data not shown). The time course for PEDF induction in this study suggests a correlation between an increase in levels of PS1 CTF in the membrane and cleavage of VEGFR-1. The PS1 CTF was visible after 30 min of PEDF stimulation with appearance of the 80-kDa...
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C-terminal fragments of VEGFR-1 in the cytosol showing similar kinetics with a detectable increase as early as 1 h after the addition of PEDF. A challenging thought is that the 80-kDa C-terminal fragments of VEGFR-1 released into the cytosolic could act to inhibit PEDF-2 phosphorylation (34) and that some remnant 100-kDa N-terminal domains are released extracellularly to involve sequestration of the VEGF similar to the soluble form of VEGFR-1 (35).

In hemangioma endothelial cells VEGF activates VEGFR-2 via an autocrine pathway, which results in constitutive tyrosine phosphorylation, VEGF-2 and VEGFR-1 nuclear localization, and mitogen-activated protein kinase activation (36). This translocation of VEGFR-1 from the cell surface to the nucleus suggests that VEGF signaling must at least partially be dependent on its receptors nuclear localization. In a similar context, nuclear translocation of fibroblast growth factor receptors has been described in a recent study by Myer and co-workers (37). They showed that both full-length fibroblast growth factor receptors 1 and 2 accumulate within the nucleus together with fibroblast growth factor itself. Taken together it suggests that nuclear translocation of growth factor receptors has major functional importance for signal transduction and regulating the classic mitogen-activated protein kinase/extracellular signal-regulated kinase pathways.

It is well recognized that VEGF modulates theangiogenic activity of endothelial cells via receptor signaling (predominantly VEGFR-2) and cross-talk between its receptors (12–15). Here our data show that suppression of VEGFR-2 inhibits microvascular endothelial tube formation, whereas suppression of VEGFR-1 causes the microvascular endothelial cells to form disorganized tubular networks, which is consistent with previous in vivo studies that flt-1−/− mutant mice manifests vascular overgrowth and disorganization (38, 39). This is further support for the notion that VEGFR-1 impacts on angiogenesis by negatively regulating endothelial cells. However, that suppression of VEGFR-2 was unable to rescue impeded VEGF-induced tubule formation by PEDF suggests that the inhibitory effect of PEDF on tubule formation may not directly relate to VEGFR-2. There is now considerable evidence that VEGFR-1 can also act as a positive regulator of angiogenesis. We and others have shown that PIGF, which is specific for VEGFR-1, is able to sustain in vitro angiogenesis and to promote pathological angiogenesis (16, 40). Our findings that PEDF inhibits VEGF-induced angiogenesis via γ-secretase cleavage of VEGFR-1 provide unprecedented evidence that VEGFR-1 may regulate VEGF-2 signaling through different but parallel pathways.

As for other receptor tyrosine kinases, tyrosine kinase activity appears to be crucial for VEGFR-1 function, and the autophosphorylation status appears to indicate the level of tyrosine kinase activity of VEGFR-1 (21, 41). Not surprisingly, we also showed that there is an increase in autophosphorylation of VEGFR-1 in response to VEGF and that these changes are not due to alterations in overall VEGFR-1 expression. Furthermore, deletion of the tyrosine kinase domain of VEGFR-1 and inhibition of tyrosine kinase activity impairs pathological angiogenesis (42, 43). We observed that PEDF is able to significantly reduce the phosphorylation of VEGFR-1 both in the presence and the absence of VEGF. Furthermore, VEGF-induced autophosphorylation of VEGFR-1 is reduced as early as 1 h after the addition of PEDF, suggesting that PEDF inhibits acute phosphorylation of VEGFR-1. Whether VEGFR-1 phosphorylation represents a signaling pathway or is in some way implicated in regulating γ-secretase cleavage of VEGFR-1 cannot be answered from this study.

The phosphorylation and nuclear localization of VEGFR-1 in control cells may indicate a homeostatic mechanism or simply represent low level autocrine secretion of VEGF. Autocrine secretion of VEGF by vascular endothelia has previously been reported (44). The mechanism by which PEDF exerts its effect is unclear largely because of the difficulties in identifying its receptor. A putative 85-kDa receptor for PEDF has been identified on neural cells responsible for the neurotropic properties of PEDF (45). However, this receptor has not been identified on vascular endothelial cells or shown to regulate angiogenesis. Although an as yet unidentified receptor remains a possibility, considerable research effort in academia and industry has surprisingly failed to identify such a receptor, suggesting that PEDF may signal via a nonclassical route. It is possible that PEDF may in some way interact with one of the four domains making up the γ-secretase complex (46). With the identification of a 14-mer fragment of PEDF with angiogenic
activity (47), it may be possible to use a bioinformatics approach to identify the PEDF-binding site.

In summary, we have identified that VEGFR-1 plays a critical role in the regulation of angiogenesis and that PEDF acts as a potent inhibitor of VEGF-induced angiogenesis by both translocating the intracellular domain of VEGFR-1 and regulating the phosphorylation of VEGFR-1. This study has provided a novel insight into the molecular pathway by which PEDF regulates activated endothelial cell behavior and provides potential targets for medical intervention to regulate pathological angiogenesis.

REFERENCES

1. Tombran-Tink, J., and Johnson, L. V. (1989) *Investig. Ophthalmol. Vis. Sci.* 30, 1700–1707.
2. Tombran-Tink, J., Mazuruk, K., Rodriguez, I. R., Chung, D., Linker, T., Englander, E., and Chader, G. J. (1996) *Mol. Vis.* 2, 11.
3. Steele, F. R., Chader, G. J., Johnson, L. V., and Tombran-Tink, J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 1526–1530.
4. Dawson, D. W., Volpert, O. V., Gillis, P., Crawford, S. E., Xu, H., Benedict, W., and Bouch, N. P. (1999) *Science* 285, 245–248.
5. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 518–522.
6. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
7. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
8. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
9. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
10. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
11. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
12. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
13. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
14. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
15. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
16. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
17. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
18. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
19. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
20. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
21. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
22. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
23. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
24. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
25. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
26. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
27. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
28. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
29. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
30. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
31. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
32. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
33. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
34. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
35. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
36. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
37. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
38. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
39. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
40. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
41. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
42. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
43. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
44. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
45. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
46. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
47. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
48. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
49. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
50. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
51. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
52. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
53. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
54. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
55. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
56. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
57. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
58. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
59. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
60. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
61. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
62. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
63. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
64. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
65. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
66. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
67. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
68. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
69. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.
70. Struhl, G., and Greenwald, I. (1999) *Nature* 398, 522–525.