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Peptides Encoded by Exon 6 of VEGF Inhibit Endothelial Cell Biological Responses and Angiogenesis Induced by VEGF

Haiyan Jia,*,†,[1] Sylvie Jezequel,*,† Marianne Löhr,‡ Shaheda Shaikh,‡ Dana Davis,‡ Shay Soker,§ David Selwood,‡ and Ian Zachary*,[1]

**Department of Medicine and †Ark Therapeutics Limited, The Rayne Institute, University College London, 5 University Street, London WC1E 6JJ, United Kingdom; ‡Wolfson Institute for Biomedical Research, University College London, London, United Kingdom; and §Children's Hospital, Harvard Medical School, Boston, Massachusetts 02115*

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VEGF induces pathological angiogenesis and is an important target for the development of novel antiangiogenic molecules. In this study, we tested synthetic peptides based on the sequence of $VEGF_{189}$ for their ability to inhibit VEGF receptor binding and biological responses. We identified 12-amino acid peptides derived from exon 6 that inhibited VEGF binding to HUVECs, VEGF-stimulated ERK activation, and prostacyclin production. These peptides inhibited VEGF-induced mitogenesis, migration, and VEGF-dependent survival of endothelial cells, but caused no increase in apoptosis in the absence of VEGF. Exon 6-encoded peptides also caused a marked inhibition of VEGF-induced angiogenesis *in vitro.* Studies of effects of peptides on cross-linking of VEGF to its receptors and on binding of VEGF to porcine aortic endothelial cells expressing either KDR or neuropilin-1 showed that exon 6-encoded peptides effectively blocked the interaction of VEGF with both receptors. Exon 6-derived peptides caused release of bFGF from endothelial cells but inhibited bFGF-dependent ERK activation, cell proliferation and angiogenesis. Our findings indicate that VEGF exon 6-encoded peptides inhibit VEGF-induced angiogenesis, at least in part through inhibition of VEGF binding to KDR. In addition, exon 6-encoded peptides are also effective inhibitors of bFGF-mediated angiogenesis.

Key Words: **KDR; neuropilin-1; apoptosis; migration; ERK; prostacyclin.**

Vascular endothelial growth factor (VEGF) is an important mediator of vascular endothelial cell differentiation, proliferation and migration. VEGF-induced vasculogenesis and angiogenesis are essential for formation of the vasculature during embryogenesis and play a central role in pathophysiological neovascularization in human disease (1, 2). Five isoforms of VEGF have been identified, $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$, all arising from alternative mRNA splicing from a single VEGF gene containing 8 exons (3–5). VEGF expression is increased by hypoxia and several growth factors and cytokines, including bFGF, another potent angiogenic factor (1, 2, 6–8).

VEGF exerts its biological effects through high affinity binding to two tyrosine-kinase receptors, Flt-1 (VEGF-R1) and KDR (VEGF-R2), which are expressed in most vascular endothelial cells. KDR binds VEGF with lower affinity than Flt-1, and is also recognised by VEGF-C, VEGF-D, and VEGF-E (9–13). Flt-1 is the only VEGF receptor in monocytes, and is also recognised by P1GF and VEGF-B (14, 15). Neuropilin-1 (NP-1), a receptor for the semaphorin/collapsin family of polypeptides implicated in axon guidance, has recently been identified as a novel non-tyrosine-kinase receptor for $VEGF_{165}$, P1GF, VEGF-B, and VEGF-E (12, 16–18). NP-1 binds to $VEGF_{165}$ with an affinity similar to that for KDR, and is expressed in some tumour cells and endothelial cells. After binding and activation of KDR, VEGF stimulates ERK activation and an array of other early signalling events followed by short- and long-term cellular biological effects including production of prostacyclin (PGI_2) and nitric oxide, increased cell survival, cell migration, proliferation and angiogenesis (1, 2, 19–24). Flt-1 is essential for angiogenesis, but its function in the endothelium is unclear (25).

Determination of the crystal structure and site-directed mutagenesis of VEGF 8-109 (derived from ex-

Abbreviations used: bFGF, basic fibroblast growth factor; DSS, disuccinimidyl suberate; ERK1,2, extracellular signal-regulated kinases 1 and 2; HUVEC, human umbilical vein endothelial cells; NP-1, neuropilin-1; PAE/KDR, porcine aortic endothelial cells expressing KDR; PAE/NP-1, porcine aortic endothelial cells expressing NP-1; PGI_2, prostacyclin; SDS–PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor.

[1] To whom correspondence may be addressed. Haiyan Jia: Fax: 020-7679-6212. E-mail: haiyan.jia@ucl.ac.uk.. Ian Zachary: Fax: 020-7679-6212. E-mail: i.zachary@ucl.ac.uk

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ons 2–5) has identified residues crucial for receptor binding (26, 27). A putative binding site for NP-1 has been localised to the exon 7-encoded domain (16, 28). The exon 6-encoded region is strongly implicated in binding to heparin and it was previously reported that peptides corresponding to this region induced release of cell-associated bFGF via inhibition of bFGF binding to heparin sulphate proteoglycans (29).

Because VEGF-mediated angiogenesis has been shown to underly the pathology of solid tumour growth and other neovascular diseases such as diabetic retinopathy, rheumatoid arthritis, and psoriasis (30–33), much effort has been directed towards identifying inhibitors of the VEGF/receptor interaction (34, 35). The purpose of the present study was to identify VEGF-derived peptides which modulate VEGF receptor binding and biological activities. VEGF-derived peptides based on the $VEGF_{189}$ sequence encoded by exons 1–8 were synthesised and tested in cultured human umbilical vein endothelial cells (HUVEC) to determine their effect on VEGF receptor binding. Selected peptides were further tested to investigate their effects on VEGF-mediated biological functions. The results show that VEGF peptides encoded by exon 6 inhibit radiolabelled VEGF binding to HUVECs and to KDR or NP-1. These peptides also inhibit cell proliferation, migration, survival and angiogenesis induced by VEGF. Furthermore, exon 6-encoded peptides are also effective inhibitors of angiogenesis and other biological responses induced by bFGF.

MATERIALS AND METHODS

Materials. Recombinant human $VEGF_{165}$ and bFGF were obtained from R & D Systems. Collagenase, collagen type I, and DSS were purchased from Sigma. M199 and Ham's F12 medium were from Life Technologies. EBM and DMEM/25 mM HEPES, pH 7.3 were from Clonetics and Sigma, respectively. All other reagents used were of the purest grade available.

Peptide synthesis. All peptides were synthesised on an automated AMS 422 Multiple Peptide Synthesiser using the solid phase approach. The Rink Amide MBHA resin (0.59 and 0.68 mmol/g loading) and the N-Fmoc strategy with orthogonal protection (acetamidomethyl, tert-butyl) of the Cys side chains of derivatives to be cyclised were applied. The crude peptides were analysed by analytical LC-MS on a Quattro LC Mass Spectrometer from Micromass with a Hewlett-Packard HPLC instrument model 1100, and purified by preparative reverse-phase HPLC (Gilson), monitored at 215 nm and eluted at a flow rate of 20 ml/min. Amino acid analysis was performed at the University of Cambridge, Protein and Nucleic Acid Chemistry Facility, and all peptides had expected masses. All peptides used for experimental purposes were >90% pure.

Cell culture. HUVECs were prepared from fresh human umbilical cords by collagenase digestion (0.25 mg/ml, 37°C, 10 min) and cultured in endothelial cell basal medium (EBM) supplemented with 10% fetal bovine serum, 10 ng/ml human epidermal growth factor, 12 μg/ml bovine brain extract, 50 μg/ml gentamicin sulfate, and 50 ng/ml amphotericin-8.

Porcine aortic endothelial cells expressing NP-1 (PAE/NP-1) (16) were provided by Dr. Shay Soker. The cells were grown in Ham's F12 medium containing 10% fetal bovine serum and 25 μg/ml hygromycin B. PAE cells expressing KDR (PAE/KDR) (36) were provided by Professor Lena Claesson-Welsh and grown in Ham's F12 medium containing 10% fetal bovine serum and 250 μg/ml Gentamicin G418.

^{125}I-$VEGF_{165}$ binding. Confluent endothelial cells in 24-well plates were washed twice with PBS. At 4°C various concentrations of peptides diluted in binding medium (DMEM, 25 mM HEPES pH 7.3 containing 0.1% BSA) were added, followed by addition of the indicated concentration of ^{125}I-$VEGF_{165}$ (1200–1800 Ci/mmol, Amersham). After 2 h of incubation at 4°C, the medium was aspirated and washed 4 times with cold PBS. The cells were lysed with 0.25 M NaOH, 0.5% SDS solution, and the bound radioactivity of the lysates was measured. Non-specific binding was determined in the presence of 100-fold excess unlabelled $VEGF_{165}$.

Cross-linking and immunoprecipitation. Confluent HUVECs were bound with ^{125}I-$VEGF_{165}$ at 4°C for 2 h as described above and then washed 3 times with PBS. The bound ^{125}I-$VEGF_{165}$ was cross-linked to the cells by incubation with 1.5 mM disuccinimidyl suberate (DSS) for 20 min at room temperature. After 3 washes with PBS at 4°C, the cells were solubilised in lysis buffer (64 mM Tris–HCl, pH 6.8, 0.2 mM Na_3VO_4, 2% SDS, 10% glycerol, 0.1 mM 4-(2-Aminoethyl)benzenesulfonyl fluoride (AEBSF), 5 μg/ml leupeptin) and scraped off the plates. After centrifugation at 16,000*g* for 20 min at 4°C, cross-linked ^{125}I-$VEGF_{165}$-receptor complexes were subjected to 7.5% SDS–PAGE. Gels were dried and exposed to X-ray film.

Immunoprecipitation of cross-linked complexes were performed by lysing cells which had been cross-linked with ^{125}I-$VEGF_{165}$ with a buffer containing 1% Triton X-100, 150 mM NaCl, 10 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA pH 8.0, 0.2 mM Na_3VO_4, 0.2 mM AEBSF, and 0.5% NP-40 for 30 min on ice. The cell lysate was collected and centrifuged at 16,000*g* for 15 min to remove insoluble material. Anti-NP-1 antibodies were added to the lysates, and following 1 h of incubation at 4°C, 20 μl of Protein A-agarose was added for an additional 30 min at 4°C. Protein A-agarose beads were pelleted at 16,000*g* for 4 min, washed 3 times with immunoprecipitation buffer, and resuspended in SDS–PAGE sample buffer. The samples were subjected to 7.5% SDS–PAGE. Gels were dried and exposed to X-ray film.

Measurements of bFGF and VEGF. The concentrations of VEGF or bFGF were determined in cell culture media after various treatments using specific immunoassay kits (R & D Systems) according to the manufacturer's instructions.

ERK activation assay. Cells were pretreated with peptides for 15 min followed by treatment with growth factors for 10 min, and cells were immediately extracted by lysis buffer (64 mM Tris–HCl, pH 6.8, 0.2 mM Na_3VO_4, 2% SDS, 10% glycerol, 0.1 mM AEBSF, 5 μg/ml leupeptin). ERK activation was determined by immunoblotting cell extracts with an antibody which specifically recognises ERK 1 and 2 activated by phosphorylation at Thr^{202} and Tyr^{204} (23). Immunoreactive bands were visualised by chemiluminescence using horseradish peroxidase-conjugated anti-rabbit IgG and ECL reagent.

PGI_2 assay. After cell treatments, the PGI_2 content of the cell supernatants was measured by enzyme-immunoassay of its stable metabolite, 6-keto-prostaglandin $F_{1\alpha}$, using a kit (Amersham) according to the manufacturer's instructions.

DNA synthesis and cell proliferation. DNA synthesis was determined by measurement of 5-bromo-2′-deoxy-uridine (BrdU) incorporation. HUVECs were seeded at a density of 8000 cells per well in 96-well plates. After 24 h incubation the medium was replaced with fresh EBM containing 2% FBS and 10 ng/ml $VEGF_{165}$ or 10 ng/ml bFGF in the absence or presence of selected peptides and incubated for 18 h. After this time 10 μM BrdU was added to the cultures and they were incubated for a further 6 h. DNA synthesis was then measured by BrdU uptake using a BrdU detection kit (Boehringer Mannheim) according to the manufacturer's instructions.

For cell proliferation assays, HUVECs were seeded at a density of 4000 cells per well in 96-well plates. Twenty-four hours after plating,

the medium was replaced with fresh EBM containing 0.5% FBS and 10 ng/ml $VEGF_{165}$ or 10 ng/ml bFGF in the absence or presence of selected peptides. After 4 days, cells were stained with Pro-Diff (Braidwood Laboratories, Beckenham, Kent, UK) and the cell numbers were determined in 12 fields per well using an eyepiece indexed graticule at ×100 magnification.

Cell migration. Cell migration was measured in a modified Boyden chemotaxis chamber (NeuroProbe Inc., Cabin John, MD) as previously described (24). VEGF in M199/1% BSA was placed in the bottom wells of the chamber. Polycarbonate filters with 8-μm pores (Osmonics Poretics) were placed between the bottom and top chambers. Cells were trypsinised, washed, and resuspended in M199/1% BSA to give a final cell concentration of 3×10^5/ml. 15,000 cells with or without peptides as indicated were placed into each well of the top chamber, and the chemotaxis chambers were incubated at 37°C for 6 h. After the incubation, unmigrated cells were removed from the top side of the filters, and migrated cells were stained with Pro-Diff. The stained cells from each well were counted at ×100 magnification using an eyepiece indexed graticule.

Apoptosis. Subconfluent HUVECs in 6-well plates were washed twice with EBM free of serum and other supplements, and incubated with the indicated additions for 24 h. The cells were then trypsinised, collected by centrifugation, and stained with fluorescein-conjugated annexin V and propidium iodide (Boehringer Mannheim). After staining, the cells were analysed by flow cytometry using a FACScan (Becton Dickinson). Annexin V-positive staining cells are apoptotic cells. Cells which were propidium iodide-positive and annexin V-negative were not counted as apoptotic cells.

In vitro angiogenesis. An *in vitro* coculture assay of human endothelial cell-derived tubule formation (TCS Biologicals) was used to investigate the effects of selected peptides on angiogenesis. The peptides, together with $VEGF_{165}$ were added at the start of the experiment and at each medium change at days 4, 7, and 9. At day 11, the cultures were fixed with 70% ethanol for 30 min at room temperature and subsequently immunostained for the endothelial cell adhesion molecule CD31. Fixed cells were incubated with the primary antibody (mouse anti-human CD31) for 1 h at 37°C, washed 3 times in blocking buffer (PBS containing 1% BSA), and then incubated with the secondary antibody (goat anti-mouse IgG conjugated to alkaline phosphatase) for 1 h at 37°C. After 3 washes in distilled water, the cells were finally incubated with the substrate solution of alkaline phosphatase for 10 min at 37°C. Immunostaining was observed and photographed using an inverted microscope (Zeiss Axiovert CFL25) fitted with a ×4 objective lens. In each experiment, control wells with either $VEGF_{165}$ alone, $VEGF_{165}$ plus suramin, or no addition were included.

Endothelial cell-derived tubules were quantified as described (37) using the Chalkley point counting technique with a 25-point Chalkley point eyepiece graticule under low power (×40 magnification, 12 fields per well). The number of Chalkley points on the graticule overlay with endothelial cell-derived tubules on the plate was counted.

RESULTS

Twenty amino acid VEGF-derived peptides corresponding to the sequence of $VEGF_{189}$, encoded by exons 1 to 8, were synthesised and tested for their effects on ^{125}I-$VEGF_{165}$ binding to high affinity sites in HUVECs. Peptides encoded by exons 2–3 and exons 6–7 inhibited the binding of 0.1 nM ^{125}I-$VEGF_{165}$ by greater than 55% and 75%, respectively, at concentrations of 0.1 to 1 mM (data not shown). To further localise regions of $VEGF_{189}$ which could inhibit radiolabelled ligand binding, 12-residue peptides were synthesised and tested on ^{125}I-$VEGF_{165}$ binding. As shown in Fig. 1A, peptides 121–132, 125–136 and 129–140 containing exon 6-encoded residues, peptide 133–144 containing seven residues, encoded by exon 6 an five residues of exon 7, and peptide 153–164 containing exon 7-encoded residues, effectively inhibited (>55% inhibition) the binding of 0.1 nM ^{125}I-$VEGF_{165}$. In addition, peptide 11–22 derived from exons 2–3 inhibited the binding of 0.1 nM ^{125}I-$VEGF_{165}$ by 84%. The concentration-dependence of the effects of selected 12-residue VEGF peptides on ^{125}I-$VEGF_{165}$ binding was determined (Fig. 1B, Table 1). Inhibition of ^{125}I-$VEGF_{165}$ binding by all the peptides tested was concentration-dependent and reached a maximum of 64 to 100% inhibition for peptides 11–22, 121–132, 125–136, 129–140, and 133–144. Further evaluation of peptide 11–22 was prevented by problems of low solubility and purification. Subsequent studies therefore concentrated on peptides encoded by exon 6.

Since a 16-residue exon 6-encoded peptide corresponding to 119–134 has been reported to release cell-associated bFGF (29), we tested the possibility that peptides derived from the basic region of $VEGF_{189}$ could induce release of cell-associated bFGF and/or VEGF. As shown in Fig. 2A, the 20-residue peptides 125–144 and 145–164 increased bFGF in the cell culture medium of HUVECs at 4°C by 36- and 15-fold, respectively, above the untreated control. The 12-residue peptide 125–136 induced a significant but less marked release of bFGF from HUVECs. In contrast, no VEGF (≥15 pg/ml) was detected in the supernatants of HUVECs following treatment by these peptides (data not shown). bFGF at concentrations up to 10 nM (156 ng/ml) had no effect on ^{125}I-$VEGF_{165}$ receptor binding (data not shown).

We next determined whether peptides which inhibited ^{125}I-$VEGF_{165}$ receptor binding also affected biological responsiveness of HUVECs to $VEGF_{165}$. $VEGF_{165}$ induces PGI_2 production in HUVECs mediated via activation of ERKs 1 and 2 (22, 23) and effects of peptides on this pathway were examined. Peptides 121–132 and 125–136 did not affect basal PGI_2 production, but they inhibited $VEGF_{165}$-induced PGI_2 production by 57 and 67%, respectively, at 1 mM and by 34 and 53%, respectively, at 100 μM (Fig. 2B). Peptide 117–128 had no effect on VEGF-stimulated PGI_2 production at 100 μM, and caused a 41% inhibition at 1 mM. Pretreatment of cells for 15 min with VEGF 125–136 caused a striking reduction in ERK activation induced by addition of VEGF for a further 10 min (Fig. 2C).

Based on these findings, we examined whether 12-residue exon 6-encoded VEGF peptides could affect endothelial mitogenesis and migration. As shown in Fig. 3A, peptides 121–132 and 125–136 at 1 mM inhibited $VEGF_{165}$-stimulated DNA synthesis by 49 and 38%, respectively. At a concentration of 1 mM, peptide 165–176 which had no effect on $VEGF_{165}$ receptor bind-

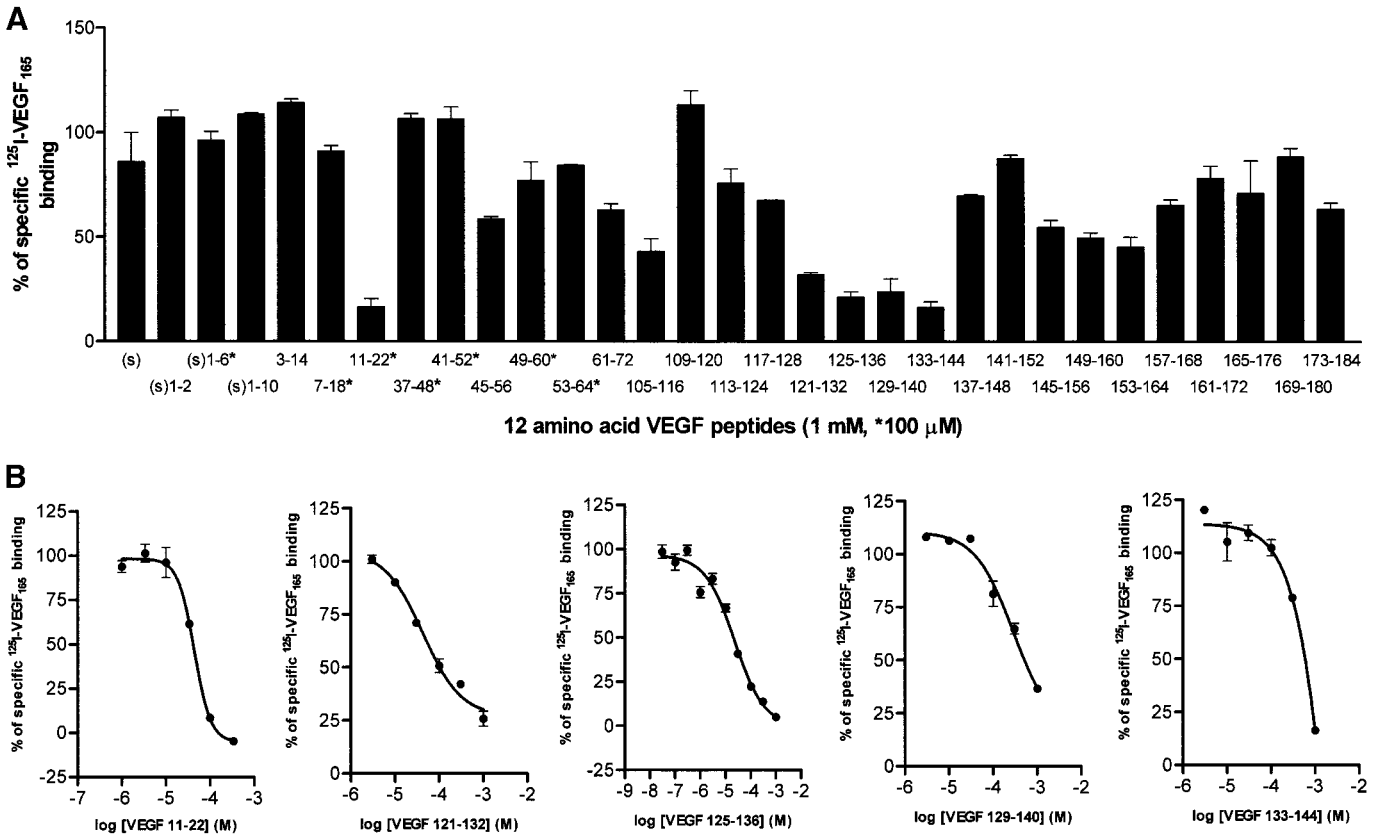

FIG. 1. Effects of 12 amino acid VEGF peptides on ^{125}I-VEGF$_{165}$ binding to HUVECs. (A) Confluent HUVECs were incubated with ^{125}I-VEGF$_{165}$ at 0.1 nM for 2 h at 4°C in the presence of 12 amino acid VEGF peptides at 1 mM unless otherwise indicated as described under Materials and Methods. Bars represent mean percentages ± SEM of specific ^{125}I-VEGF$_{165}$ binding calculated from the results of two or three independent experiments each performed in triplicate. The specific ^{125}I-VEGF$_{165}$ binding in the absence of any peptide is 100%. Some peptides were used at 100 μM (*) due to their low solubility. Peptides (s), (s)1–2, (s)1–6, and (s)1–10 contained signal sequence-derived residues. (B) Inhibition of ^{125}I-VEGF$_{165}$ binding by selected 12 amino acid VEGF peptides. Confluent HUVECs were incubated with ^{125}I-VEGF$_{165}$ at 0.1 nM in the presence of the indicated concentrations of 12 amino acid VEGF peptides. Values represent mean percentages ± SEM of specific ^{125}I-VEGF$_{165}$ binding calculated from triplicate determinations. Similar results were obtained from two or three independent experiments.

ing (Fig. 1A) did not inhibit mitogenesis in response to VEGF$_{165}$ (Fig. 3A). VEGF 125–136 partially inhibited VEGF-induced cell proliferation by 18% at 100 μM and by 38% at 1 mM (Fig. 3B). VEGF 125–136 caused a more marked inhibition of VEGF-induced endothelial cell chemotaxis with 41% inhibition at 100 μM and a 68% reduction at 1 mM (Fig. 3C).

TABLE 1

The Sequences and IC$_{50}$s for Inhibitory 12 Amino Acid VEGF Peptides

Peptide	Sequence	IC$_{50}$ (μM)
11–22	HHEVVKFMDVYQ	42
121–132	KGKGQKRKRKKS	100
125–136	QKRKRKKSRYKS	20
129–140	RKKSRYKSWSVP	520
133–144	RYKSWSVPCGPC	600

Note. The amino acid sequences of 12-residue VEGF peptides are shown. The IC$_{50}$ for each peptide is equivalent to the concentration required to inhibit 0.1 nM of ^{125}I-VEGF$_{165}$ binding to HUVECs by 50%.

To determine the effects of exon 6-encoded peptides on VEGF-mediated cell survival, apoptosis was measured by annexin V staining. As shown in Fig. 3D, serum deprivation of HUVECs for 24 h strikingly increased the frequency of apoptotic cells (30%) compared to continuous incubation in 10% serum (6% apoptotic cells). VEGF$_{165}$ markedly reduced the apoptotic effect of serum starvation (13% apoptotic cells). Incubation with peptide 125–136 alone for 24 h in the absence of VEGF$_{165}$ did not increase the frequency of apoptotic cells compared with the effect of serum deprivation, indicating that the exon 6-encoded peptide itself has no apoptotic effect. Indeed, it was noted that peptide 125–136 by itself decreased HUVEC apoptosis compared with serum withdrawal in the absence of peptide. In contrast, incubation with peptide 125–136 inhibited VEGF$_{165}$-mediated anti-apoptotic activity, reducing it to near the level obtained with the peptide alone.

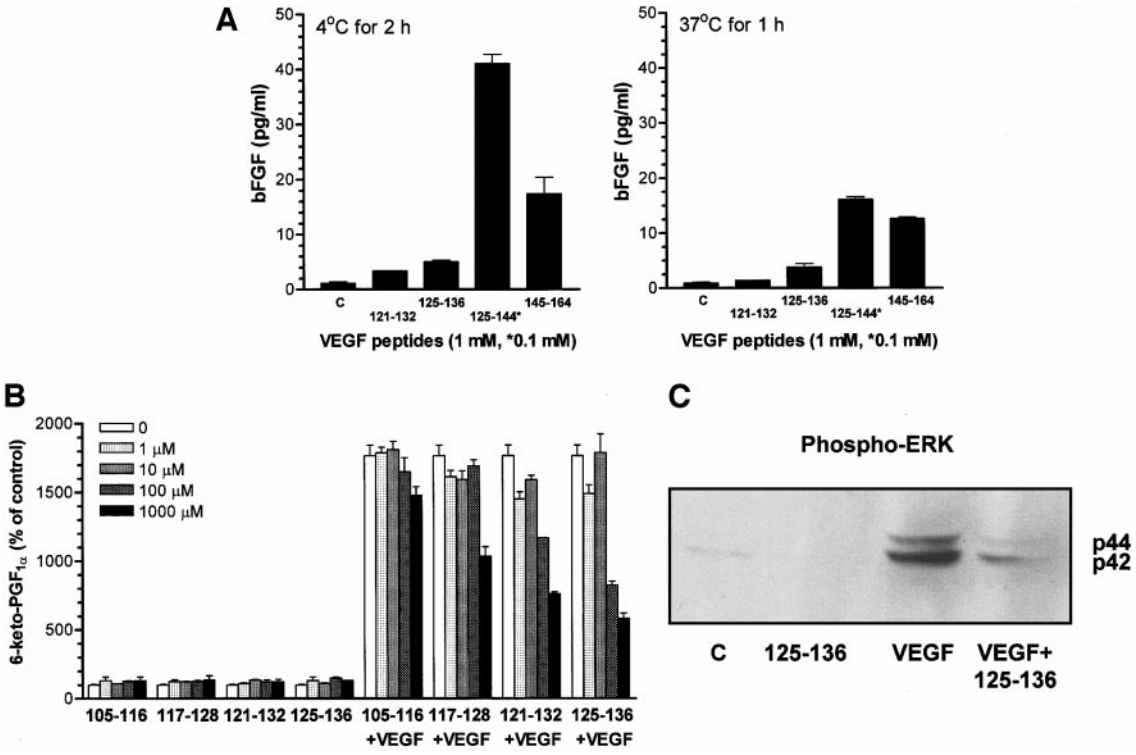

FIG. 2. Release of cell-associated bFGF by VEGF exons 6 and 7-derived peptides and inhibition of VEGF-stimulated ERK activation and PGI_2 production by VEGF exon 6-encoded peptides. (A) Confluent HUVECs in 12-well plates were incubated for 2 h at 4°C and 37°C for 1 h, respectively, without (C, control) or with the peptides at the indicated concentrations, and supernatants were collected for bFGF measurement. Bars represent means ± SEM of bFGF (pg/ml) obtained from duplicate determinations. (B) Confluent HUVECs in 12-well plates were preincubated with the peptides at the indicated concentrations for 30 min and then either incubated for a further 30 min with no further addition or were exposed to 25 ng/ml VEGF for 1 h. Some cells were incubated without peptides either in the absence or presence of 25 ng/ml VEGF. The supernatants were collected and 6-keto-prostaglandin $F_{1\alpha}$ was measured by enzyme immunoassay. Bars represent mean percentages ± SEM of the unstimulated control level of 6-keto-prostaglandin $F_{1\alpha}$ (100%) obtained from duplicate determinations. Similar results were obtained from two independent experiments. (C) Confluent HUVECs in 6-well plates were either untreated (C, control) or pretreated for 15 min with 1 mM peptide 125–136, and then treated with 25 ng/ml VEGF for 10 min. Cell extracts were prepared, and ERK activation was determined by Western blotting as described under Materials and Methods.

Peptides 121–132 and 125–136 were next evaluated for their biological effects on an *in vitro* assay of endothelial cell-derived tubule development. As visualised by immunostaining for the endothelial cell adhesion molecule CD31, some endothelial cell-derived tubules developed in the untreated control (Fig. 4B). Production of VEGF increased over the time-course of the angiogenesis assay in these untreated control cells suggesting that endothelial cell-derived tubule formation in the absence of exogenous $VEGF_{165}$ is most likely due to endogenous VEGF production (Fig. 4A). Exogenous $VEGF_{165}$ (10 ng/ml) enhanced tubule formation as indicated by an increase in the number and length of tubules and an increased number of branch points, resulting in a network of branching tubes (Fig. 4E). Quantification of these changes showed that exogenous $VEGF_{165}$ induced a 1.8-fold increase in endothelial cell tubules (Fig. 4H). At a concentration of 1 mM, peptide 121–132 inhibited angiogenesis as indicated by a decrease in the number and length of endothelial cell-derived tubules (Figs. 4C and 4H) compared to the untreated control. A characteristic feature of the effect of peptide 121–132 was the formation of large endothelial cell islands. Peptide 125–136 was more markedly anti-angiogenic with fewer visible tubules and smaller endothelial cell islands compared with peptide 121–132 (Fig. 4D), and an almost complete inhibition of tubule formation at 1 mM (Fig. 4H). Peptides 121–132 and 125–136 at 1 mM effectively suppressed the angiogenic response induced by exogenous $VEGF_{165}$ with 125–136 more effective than 121–132 (Figs. 4F, 4G, and 4H). As shown in Fig. 4H, peptide 125–136 at 100 μM blocked tubule formation induced by exogenous $VEGF_{165}$. Peptide 117–128, which weakly inhibited ^{125}I-$VEGF_{165}$ receptor binding and VEGF-induced PGI_2 production, had little detectable effect on control and VEGF-induced angiogenesis at 1 mM (data not shown).

To identify the specific ligand-receptor interactions inhibited by exon 6-encoded peptides we examined their effects on cross-linking of ^{125}I-$VEGF_{165}$ to its receptors in HUVECs. In agreement with previous findings (28, 38), covalent cross-linking of bound ^{125}I-

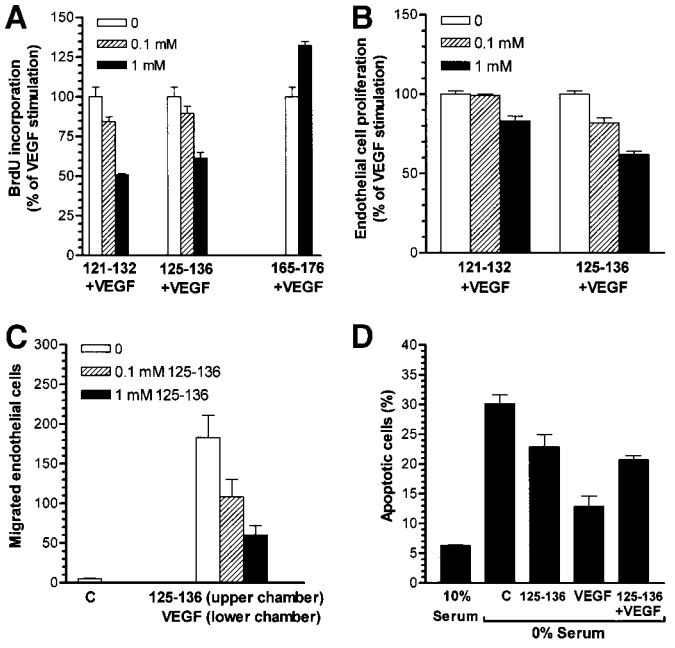

FIG. 3. Inhibition of VEGF-mediated cell mitogenesis, migration and survival by VEGF exon 6-encoded peptides. (A) HUVECs seeded on 96-well plates were stimulated by addition of 10 ng/ml VEGF in the absence or presence of the peptides at the indicated concentrations for 24 h. BrdU incorporation was determined as described under Materials and Methods. Bars represent mean percentages ± SEM of BrdU incorporation stimulated by VEGF calculated from triplicate determinations. VEGF induced a 1.5 ± 0.1-fold increase in BrdU incorporation above unstimulated controls. Similar results were obtained from two independent experiments. (B) Cell proliferation stimulated by addition of 10 ng/ml VEGF in the absence or presence of the peptides at the indicated concentrations was determined as described under Materials and Methods. Bars represent mean percentages ± SEM of cell proliferation stimulated by VEGF calculated from triplicate determinations. VEGF induced a 3.0 ± 0.4-fold increase in cell numbers above unstimulated controls. Similar results were obtained from three independent experiments. (C) M199 in the absence (C, control) or presence of 25 ng/ml VEGF was placed in the bottom wells of a 48-well chemotaxis chamber. 15,000 cells in the absence or presence of peptide 125–136 at concentrations as indicated were placed in the top wells of the chamber. After 6 h, migrated cells were stained and counted as described under Materials and Methods. Bars represent means ± SEM of migrated cells counted from quadruplicate determinations. Similar results were obtained from three independent experiments. (D) Subconfluent HUVECs were incubated with either 10% serum or serum free EBM containing 1 mM peptide 125–136, 25 ng/ml $VEGF_{165}$, or 25 ng/ml $VEGF_{165}$ in the presence of 1 mM peptide 125–136 for 24 h. The frequency of apoptotic cells was determined by flow cytometric analysis of annexin V and propidium iodide staining as described under Materials and Methods. Bars represent mean frequencies ± SEM of apoptotic cells calculated from the results of three independent experiments each performed in duplicate.

$VEGF_{165}$ to HUVECs consistently produced 2 radiolabelled bands in SDS–PAGE gels of 260 and 160 kDa (Fig. 5), corresponding to complexes of ^{125}I-$VEGF_{165}$ with KDR and NP-1, respectively. A third band of 220 kDa corresponding to Flt-1-^{125}I-$VEGF_{165}$ complexes was detected much more weakly. Immunoprecipitation of lysates of cross-linked complexes of HUVECs by specific anti-NP-1 antibodies produced a major radiolabelled band of 160-kDa which co-migrated with the 160-kDa radiolabelled band detected in non-immunoprecipitated whole cell lysates of cross-linked complexes (Fig. 5A). The formation of the 160 kDa ^{125}I-$VEGF_{165}$-NP-1 complex was selectively abolished by peptide 121–132 at 10 μM and peptide 125–136 at 3 μM, respectively, whereas labelling of the 260-kDa band (KDR) was reduced, but not abolished in the presence of 1 mM peptide (Figs. 5C and 5D). Cross-linking of ^{125}I-$VEGF_{165}$ was unaffected by peptide 3–14 at 1 mM (Fig. 5B), a concentration which had no inhibitory effect on VEGF receptor binding (Fig. 1A). VEGF receptor specificity of exon 6-encoded peptide 125–136

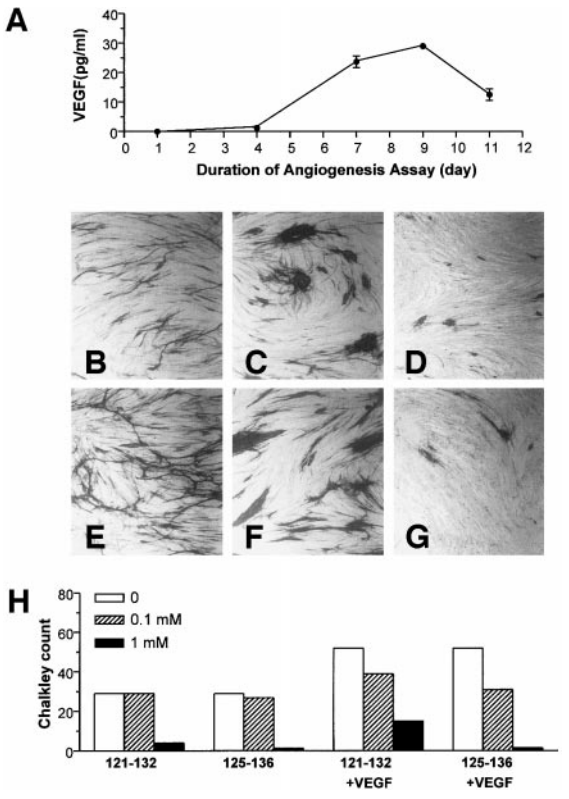

FIG. 4. Inhibition of VEGF-induced *in vitro* angiogenesis by VEGF exon 6-encoded peptides. Human endothelial cells were co-cultured with human fibroblasts in a 24-well plate over a period of 11 days. The medium was changed every 3 days, and the conditioned medium was collected for immunoassays. The control factors and/or test peptides were added at the start of the experiment (day 1) and at each medium change (days 4, 7, and 9). Endothelial tubulogenesis was visualised by immunostaining for CD31 on day 11 and culture wells were photographed at ×40 magnification. (A) VEGF released from untreated control during 11 days was measured by specific immunoassay. (B) Untreated control; (C) 1 mM peptide 121–132; (D) 1 mM peptide 125–136; (E) 10 ng/ml VEGF; (F) 10 ng/ml VEGF plus 1 mM peptide 121–132; and (G) 10 ng/ml VEGF plus 1 mM peptide 125–136. (H) Quantitative analysis of effects of VEGF peptides at concentrations as indicated on endothelial cell-derived tubules. The tubule density was measured by the Chalkley point counting method as described under Materials and Methods.

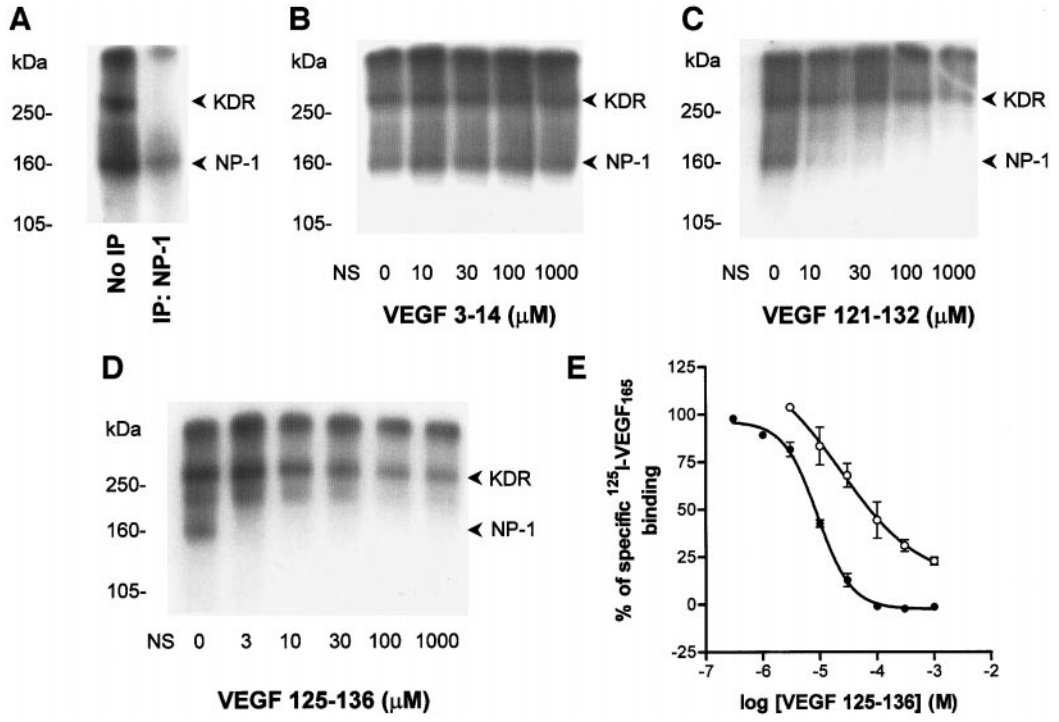

FIG. 5. Inhibition of cross-linking of ^{125}I-VEGF$_{165}$ to its receptors and ^{125}I-VEGF$_{165}$ binding to PAE/NP-1 or PAE/KDR cells by VEGF exon 6-encoded peptides. (A) ^{125}I-VEGF$_{165}$ at 0.1 nM was bound and cross-linked to HUVECs. The cells were lysed and some lysates directly analysed by 7.5% SDS–PAGE (No IP) and other lysates were immunoprecipitated with anti-NP-1 antibodies (IP: NP-1). The immunocomplexes were resolved by 7.5% SDS–PAGE as described under Materials and Methods. (B–D) ^{125}I-VEGF$_{165}$ at 0.1 nM was bound to HUVECs in the presence of increasing concentrations of peptide 3–14 (B); 121–132 (C); or 125–136 (D). The bound ^{125}I-VEGF$_{165}$ was cross-linked to HUVECs. The cells were lysed and proteins were resolved by 7.5% SDS–PAGE as described under Materials and Methods. NS indicates non-specific binding which was determined in the presence of 10 nM unlabelled VEGF$_{165}$. (E) Confluent PAE/NP-1 (black circles) or PAE/KDR (open circles) cells were incubated with ^{125}I-VEGF$_{165}$ at 0.2 nM for 2 h at 4°C in the presence of peptide 125–136 at the indicated concentrations as described under Materials and Methods. Values represent mean percentages ± SEM of specific ^{125}I-VEGF$_{165}$ binding calculated from triplicate determinations. Similar results were obtained from two independent experiments.

was further evaluated in PAE cells expressing either NP-1 or KDR. Peptide 125–136 inhibited ^{125}I-VEGF$_{165}$ binding to either PAE/NP-1 or PAE/KDR in a concentration-dependent manner (Fig. 5E). Inhibition of binding reached a maximum of 100% at 100 μM peptide 125–136 in PAE/NP-1 and 77% at 1 mM in PAE/KDR. Half-maximal inhibition of binding of ^{125}I-VEGF$_{165}$ to PAE/NP-1 or PAE/KDR was obtained at concentrations of 8 μM and 75 μM, respectively.

The ability of exon 6-derived VEGF peptides to release bFGF from HUVECs raised the possibility that these peptides could also modulate angiogenesis mediated by bFGF. Addition of exogenous bFGF to angiogenesis assays was accompanied by a marked increase in VEGF production during the course of the assay (Fig. 6A). bFGF simulated angiogenesis causing a 2.2-fold increase in tubule formation (Figs. 6D and 6F). Treatment with bFGF in the presence of VEGF 125–136 resulted in a marked inhibition of tubulogenesis and the formation of endothelial islands (Figs. 6E and 6F). To investigate whether VEGF 125–136 directly inhibited bFGF-dependent biological responses, the effects of this peptide on bFGF-induced ERK activation and mitogenesis were examined. As shown in Fig. 6, VEGF 125–136 almost completely abolished ERK activation induced by a 10-min treatment with bFGF (Fig. 6G) and markedly inhibited bFGF-induced DNA synthesis and proliferation (Figs. 6H and 6I).

DISCUSSION

In the present study, we have demonstrated for the first time that peptides encoded by VEGF exon 6 inhibit VEGF biological effects on vascular endothelial cells including ERK activation, endothelial production of PGI_2, mitogenesis, migration and survival of endothelial cells, and angiogenesis *in vitro.* VEGF exon 6-encoded peptides also had striking inhibitory effects on bFGF-induced angiogenesis and endothelial biological responses.

VEGF 125–136 was the most potent exon 6-encoded inhibitor of VEGF receptor binding with an IC_{50} of 20 μM, compared with an IC_{50} of 100 μM for peptide 121–132 and 520 μM for peptide 129–140. Peptide 125–136 also inhibited VEGF-induced PGI_2 production and markedly reduced angiogenesis at 100 μM and 1

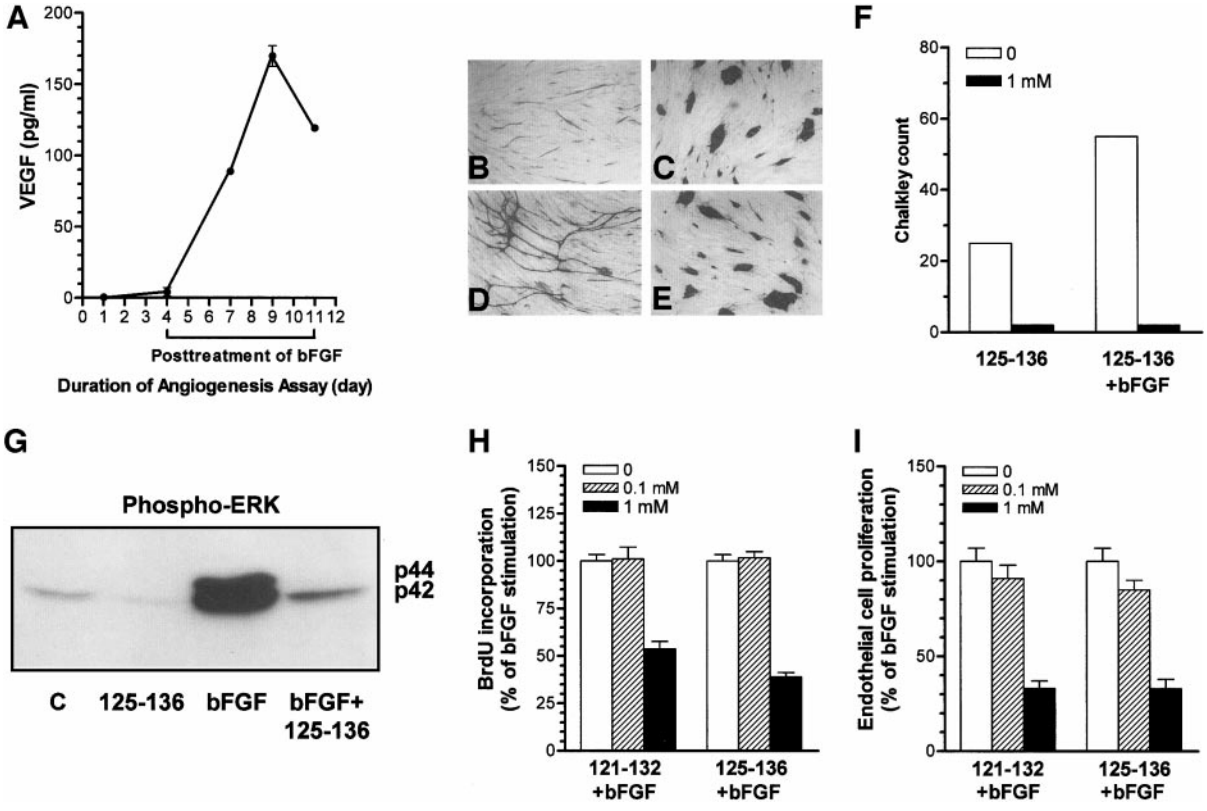

FIG. 6. Inhibition of bFGF-induced angiogenesis, ERK activation and cell proliferation by VEGF 125–136. The angiogenesis assay was performed as described in Fig. 4. The stained culture was photographed at ×40 magnification. (A) VEGF released from cells before and after bFGF treatment during 11 days was measured by specific immunoassay. (B) Untreated control; (C) 1 mM peptide 125–136; (D) 10 ng/ml bFGF; and (E) 10 ng/ml bFGF plus 1 mM peptide 125–136. (F) Quantitative analysis of effects of VEGF 125–136 at concentrations as indicated on endothelial cell-derived tubules. The tubule density was measured by the Chalkley point counting method as described under Materials and Methods. (G) Confluent HUVECs in 6-well plates were either untreated (C, control) or pretreated for 15 min with 1 mM peptide 125–136, and then treated with 25 ng/ml bFGF for 10 min. Cell extracts were prepared, and ERK activation was determined by Western blotting as described under Materials and Methods. (H) HUVECs seeded on 96-well plates were stimulated by addition of 10 ng/ml of bFGF in the absence or presence of VEGF peptides at the indicated concentrations for 24 h. BrdU incorporation was determined as described under Materials and Methods. Bars represent mean percentages ± SEM of BrdU incorporation stimulated by bFGF calculated from triplicate determinations. bFGF induced a 1.7 ± 0.1-fold increase in BrdU incorporation above unstimulated controls. Similar results were obtained from two independent experiments. (I) Cell proliferation stimulated by 10 ng/ml bFGF in the absence or presence of VEGF peptides at the indicated concentrations was determined as described under Materials and Methods. Bars represent mean percentages ± SEM of cell proliferation stimulated by bFGF calculated from triplicate determinations. bFGF induced a 3.1 ± 0.7-fold increase in cell numbers above unstimulated controls. Similar results were obtained from three independent experiments.

mM. The relative efficacies with which exon 6-encoded peptides inhibited VEGF biological responses correlated with their relative abilities to reduce VEGF receptor binding. Compared with 125–136, peptide 121–132 was a less potent inhibitor of ligand binding, and was similarly less effective in reducing PGI_2 production or angiogenesis. Peptide 117–128 was a weaker inhibitor of binding than peptide 121–132 and much less effective in inhibiting either PGI_2 production or angiogenesis. In addition, several peptides which did not inhibit VEGF receptor binding at concentrations up to 1 mM were also ineffective in inhibiting VEGF biological activities including PGI_2 production, mitogenesis, and angiogenesis. These findings indicate that the effects of exon 6-encoded peptides were not nonspecific and strongly suggest that these peptides inhibit VEGF biological responses by preventing receptor binding and activation. This conclusion is strengthened by the fact that inhibitory effects of VEGF 125–136 on angiogenesis, chemotaxis, mitogenesis and PGI_2 production occurred at 100 μM, similar to the IC_{50} of 75 μM obtained for inhibition of ^{125}I-$VEGF_{165}$ binding to PAE/KDR cells.

The antiangiogenic effects of exon 6-encoded peptides are likely to result from inhibition of proliferation, migration and cell survival induced by VEGF. It was noted, however, that while VEGF exon 6-encoded peptides had a partial inhibitory effect on VEGF-induced mitogenesis, they exhibited striking antiangiogenic effects. The more marked inhibitory effect of VEGF 125–136 on VEGF-induced chemotaxis suggests that an antimigratory effect of this peptide is a

more important mechanism underlying its ability to suppress endothelial cell tubulogenesis. The partial inhibitory effect of VEGF 125–136 on VEGF-induced proliferation may result from the generation of a functional mitogenic response at a partial level of receptor occupancy. In this context, it is noteworthy that VEGF 125–136 at 1 mM reduced VEGF binding to PAE/KDR cells by only up to 77%, and similarly cross-linking to KDR in HUVECs was incompletely blocked at this concentration. In turn, the marked effect of VEGF 125–136 on migration and tubulogenesis at 100 μM and 1 mM may reflect the dependence of these biological responses on a higher level of KDR occupancy. Importantly, this peptide by itself had no adverse effects on endothelial cell survival, indicating that its anti-angiogenic effects were not the result of a non-specific effect on cell viability.

Cross-linking experiments and binding studies in PAE/NP-1 and PAE/KDR cells indicated that VEGF 125–136 inhibited $VEGF_{165}$ binding to NP-1 approximately 10-fold more potently than it inhibited binding to KDR. Since $VEGF_{165}$ lacks the exon 6-encoded region and binds to NP-1 via the exon 7-encoded domain (28), these findings raise the possibility that the exon 6-encoded region may function as a regulatory domain which can inhibit VEGF binding to NP-1, and, at higher concentrations, KDR. The inhibitory effects of exon 6-encoded peptides on VEGF-mediated biological functions correlated more closely with inhibition of VEGF cross-linking to KDR on HUVECs and binding to PAE/KDR cells, than with inhibition of VEGF binding to NP-1. These findings suggest that inhibition of KDR binding is most likely to account for the inhibition of biological effects of VEGF by exon 6-encoded peptides, though they do not preclude a role for NP-1 in mediating other biological actions of VEGF. A high content of basic amino-acid residues is the most striking feature of exon 6 and this may be primarily responsible for the inhibitory action of VEGF exon 6-derived peptides. However, the fact that peptides 121–132 and 125–136 have the same number of charged basic residues but differed in their potency and efficacy suggests that sequence specificity may also contribute to their biological activity.

The results presented here show that VEGF 125–136 was also an effective inhibitor of bFGF-induced angiogenesis. The argument that VEGF 125–136 is a direct inhibitor of bFGF-mediated biological responses is greatly strengthened by the findings that this peptide strikingly reduced bFGF-induced ERK activation and mitogenesis. Given that VEGF exon 6-encoded peptides are likely to release cell-sequestered bFGF by inhibiting bFGF binding to heparan sulphate proteoglycans (29), and that heparan sulphate proteoglycans such as syndecan 4 are thought to play a critical role in bFGF receptor function (39), it is plausible that VEGF 125–136 inhibits bFGF-induced biological actions at least in part by blocking interactions between bFGF and heparan sulphate proteoglycan co-receptors. This possibility warrants further investigation.

Several studies have identified small peptides which either antagonise or stimulate angiogenesis. This is the first report of VEGF-derived peptides which inhibit angiogenesis, and endothelial cell functions induced by VEGF and bFGF. Further studies of VEGF exon-6 encoded peptides and their derivatives might be useful for the design of smaller, more specific, and more potent peptides that would serve as VEGF and/or bFGF antagonists in pathological angiogenesis-related disorders.

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