The Carboxyl-terminal Domain (111-165) of Vascular Endothelial Growth Factor Is Critical for Its Mitogenic Potency

(Received for publication, August 14, 1995, and in revised form, January 15, 1996)

Bruce A. Keyt, Lea T. Berleau, Hung V. Nguyen, Helen Chen, Henry Heinsohn, Richard Vandlen, and Napoleone Ferrara

From the Departments of Cardiovascular Research, Protein Chemistry, and Process Sciences, Genentech, Inc., South San Francisco, California 94080

Vascular endothelial growth factor (VEGF) is a potent and specific mitogen for endothelial cells. VEGF is synthesized and secreted by many differentiated cells in response to a variety of stimuli including hypoxia. VEGF is expressed in a variety of tissues as multiple homodimeric forms (121, 165, 189, and 206 amino acids/monomer) resulting from alternative RNA splicing. VEGF121 is a soluble mitogen that does not bind heparin; the longer forms of VEGF bind heparin with progressively higher affinity. The higher molecular weight forms of VEGF can be cleaved by plasmin to release a diffusible form(s) of VEGF. We characterized the proteolysis of VEGF by plasmin and other proteases. Thrombin, elastase, and collagenase did not cleave VEGF, whereas trypsin generated a series of smaller fragments. The isolated plasmid fragments of VEGF were compared with respect to heparin binding, interaction with soluble VEGF receptors, and ability to promote endothelial cell mitogenesis. Plasmin yields two fragments of VEGF as indicated by reverse phase high performance liquid chromatography and SDS-polyacrylamide gel electrophoresis: an amino-terminal homodimeric protein containing receptor binding determinants and a carboxyl-terminal polypeptide which bound heparin. Amino-terminal sequencing of the carboxyl-terminal peptide identified the plasmid cleavage site as Arg110-Ala111. A heterodimeric form of VEGF165/121 was isolated from partial plasmid digests of VEGF165. The carboxyl-terminal polypeptide (111-165) displayed no affinity for soluble kinase domain region (KDR) or Flk-1 tyrosine kinase (FLT-1) receptors. The various isoforms of VEGF (165, 165/110, 110, and 121) bound soluble kinase domain region receptor with similar affinity (approximately 30 pM). In contrast, soluble FLT-1 receptor differentiated VEGF isoforms (165, 165/110, 110, and 121) with apparent affinities of 10, 30, 120, and 200 pM, respectively. Endothelial cell mitogenic potencies of VEGF110 and VEGF121 were decreased more than 100-fold compared to that of VEGF165. The present findings indicate that removal of the carboxyl-terminal domain, whether it is due to alternative splicing of mRNA or to proteolysis, is associated with a significant loss in bioactivity.

In the present study, we studied the interaction with heparin to address the biologic significance of the larger molecular forms of VEGF. We isolated and characterized plasmin-generated fragments of VEGF165 and compared these fragments to native VEGF165 or VEGF121 with respect to various biochemical and biological functions. Our studies demonstrate that loss of the carboxyl-terminal domain, whether due to proteolysis or alternative splicing, correlates with a substantial decrease in endothelial cell mitogenic activity of VEGF.

EXPERIMENTAL PROCEDURES

Materials—L-[125I]iodine-125 radionuclide was purchased from DuPont NEN. Chorionic T hydride (N-chloro-p-toluenesulfonamide) and sodium metabisulfite were purchased from Aldrich. Tris and sodium phosphate salts (monobasic and dibasic) were obtained from Calbiochem and Scientific Products/Mallinkrodt, respectively. Hydrochloric acid and chloramine T hydrate (3-chloro-5,6-dichloro-1H-benzotriazole) were purchased from Pierce. Sephadex G-25, protamine sulfate, pepsin, trypsin, chymotrypsin, thrombin, elastase, and collagenase were purchased from Sigma. Dithiothreitol, dithioerythritol, and caspofungin were purchased from Calbiochem. The abbreviations used are: VEGF, vascular endothelial growth factor; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; CHO, Chinese hamster ovary; RCM, reduced and carboxymethylated; DTT, dithiothreitol; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PlGF, placental growth factor; KDR, kinase domain region; FLT-1, Flk-1 tyrosine kinase.

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Cardiovascular Research, Genentech, Inc., 460 Point San Bruno Blvd., South San Francisco, CA 94080.
Plasmin was from HyClone (Logan, UT). The construction, expression and purification of F25-1 and KDR receptor-IgG chimeras was as described by Park et al. (12).

VEGF<sub>165</sub> was formulated by gel filtration on G25 Sephadex in 125 mM NaCl, 10 mM sodium citrate at pH 6. Identity was established by SDS-PAGE, reverse phase HPLC, amino-terminal sequencing and amino acid composition.

VEGF<sub>165</sub> and VEGF<sub>121</sub> were expressed, refolded, and purified from Escherichia coli. We have recently developed a procedure for preparing unreduced VEGF from bacterial expression-transformed E. coli. Cells were lysed by sonication, and the VEGF<sub>165</sub> (or VEGF<sub>121</sub>) protein was recovered in an insoluble pellet after centrifugation. The pellet was washed with 4 x ular in 20 mM Tris buffer at pH 8 with 5 mM EDTA before solubilization by addition of 25 mM dithiothreitol to the wash buffer. The extraction was allowed to continue for 2 h with stirring at 4 °C before centrifugation to remove insoluble bacterial components. The supernatant was then dialyzed overnight against 0.4 M NaCl, 20 mM Tris-HCl, pH 8 at 4°C during which time the extracted protein was free of plasmin. Bromelain, protease K, thermolysin, and collagenase were from Boehringer Mannheim. The Carboxyl-terminal Domain (111–165) of VEGF

VEGF<sub>165</sub> was eluted from the heparin column in the void volume. After the column was washed (with 10 column volumes of PBS), the carboxy-terminal polypeptide (111–165) was eluted with 1 M NaCl, 50 mM phosphate at pH 7. The protein containing fractions were dialyzed against PBS. Purity and identity was assessed by SDS-PAGE, amino acid sequence and composition, reverse phase HPLC, and mass spectrometry.

Radiolabeling of VEGF—VEGF<sub>165</sub> was radiolabeled using a modification of the chloramine T-catalyzed iodination method described by Hunter and Greenwood (14). In a typical reaction, 50 μl of 1 M Tris-HCl, 0.01% Tween 20 at pH 7.5 was added to 5 μl of sodium iodide-125 (0.5 mCi) in a capped reaction vessel. An aliquot of VEGF (10 μg/10 μl) in 125 mM NaCl, 10 mM sodium citrate at pH 6 was added to the reaction vessel. Iodination was initiated by addition of 12.5 μl of mg/ml chloramine T in 0.1 M sodium phosphate, pH 7.5. After 60 s, iodination was terminated by addition of sodium metabisulfite (25 μl, 1 mg/ml) in 0.1 M sodium phosphate, pH 7.5. The reaction vessel was vortexed after each addition. The reaction mixture was immediately applied to a PD-10 column (G25 Sephadex) that was pre-equilibrated with 0.5% bovine serum albumin, 0.01% Tween 20 in phosphate-buffered saline. Fractions were collected and counted for iodine-125 radioactivity with a γ scintillation counter (LKB model 1277). Typically, the specific radioactivity of the iodinated VEGF was 25 ± 25 μCl/μg, which corresponded to approximately 1 × 10<sup>2–</sup>10<sup>2</sup> molecules of VEGF<sub>165</sub> dimer. There are four tyrosines in VEGF<sub>165</sub> at positions 21, 25, 39, and 45. Tryptic mapping of RCM VEGF<sub>165</sub> indicated approximately 30% and 70% of the radioactivity co-eluted with tryptic peptides T2 (amino acids 17–23) and T3 (amino acids 24–56), respectively.

Dodecyl Sulfate Gel Electrophoresis—Radiolabeled samples were either reduced, or reduced and carbamylated (RCM) prior to gel electrophoresis. The reduced samples were denatured in 3% SDS with 10 mM dithiothreitol (DTT) and heated at 37 °C for 3 min. Samples for reduction and carbamylmethylation were treated by a modified procedure of Crestfield et al. (15). Those samples were dialyzed overnight in 8 μl, 0.5 M Tris-HCl at pH 8.3 with 5 mM EDTA, then reduced with 10 mM DTT at 37 °C for 30 min. Iodoacetic acid (1 mM in 1 M NaOH) was added to a final concentration of 25 mM and incubated at 25 °C for 15 min. The alkylation reaction was quenched by addition of 25 mM DTT, followed by overnight dialysis at 4 °C with 50 mM NH₄CO₃. Electrophoretic analysis was by the method of Laemmli (16) on a SDS
Amino Acid Analysis and Ultraviolet Spectroscopy—A Beckman 6300 amino acid analyzer was used with a sodium citrate program and ninhydrin detection. Aliquots (10 μl each) of VEGF samples were hydrolyzed using constant boiling HCl for 24 h at 110 °C. Quantitation was based on the yields of alanine and leucine. VEGF samples were diluted in 125 mM NaCl, 10 mM sodium citrate at pH 6 and scanned for UV absorption from 190 to 800 nm on a Hewlett Packard 8452A diode array spectrophotometer. The molar extinction coefficient for VEGF was determined as 0.37 absorbance units for a 1 mg/ml solution at 276 nm.

Molecular Weights of VEGF Variants—Molecular weights of VEGF variants were analyzed using a Sciex API III triple quadrupole mass spectrometer. The data was obtained by scanning from 300 to 2000 Da with a 0.8-ms dwell time per mass step. A Harvard infusion pump was used to introduce the samples into the mass spectrometer at a flow rate of 3-5 μl/min. The data was collected in the data-summing mode and analyzed using MacBioSpec software.

Reverse Phase HPLC—Analytical separations of VEGF fragments were done using a Vydac C4, 5μm bead, 300-Å pore size column (4.6 × 250 mm) pre-equilibrated in 0.1% trifluoroacetic acid, 5% acetonitrile at 40 °C on a Hewlett Packard 1090 liquid chromatograph with diode array detection. Typically, an aliquot of VEGF (10 μl) containing 30 μg was injected onto the HPLC column with a flow rate of 1 ml/min. Reverse phase separation of VEGF and VEGF fragments was done with a two-step gradient from 5% to 25% acetonitrile with 0.1% trifluoroacetic acid for 20 min, followed by a shallow gradient from 25% to 33% in 32 min. The effluent was monitored for absorbance (at 210 and 280 nm).

Heparin Affinity Chromatography—Analytical and preparative affinity chromatography was performed on a HP1090 liquid chromatography system using a POROS HE2 heparin column (4.6 × 100 mm). The column was pre-equilibrated with 50 mM Na2PO4 buffer at pH 7.4 using a 0.5 ml/min flow rate at 40 °C. Samples of VEGF were injected and eluted with a salt gradient from 0 to 1 M NaCl in 45 min (25 ml/min). The effluent was monitored for absorbance at 210 and 280 nm. Fractions (0.5 ml each) were collected and further analyzed as described under “Results.”

Binding Assays with Soluble Receptors—Polystyrene 96-well breakaway microtiter plates were coated overnight at 4 °C with 100 μl of affinity-purified rabbit anti-human IgG, Fc-specific antibodies at 10 μg/ml in 50 mM Na2CO3 at pH 9.6. The microtiter plates were blocked with 200 μl of 10% fetal calf serum in phosphate-buffered saline (FBS/PBS) for 1 h at 25 °C. Blocking buffer was removed, and 100 μl of a solution containing receptor-IgG chimeric protein (FLTI-IgG or KDR-IgG) at 15 ng/ml (70 μl final concentration), 125I labeled VEGF165 from CHO cells (5000 cpm per well, 20 μl final), and cold competitor at varying concentrations in PBS with 10% FBS was added to the microtiter wells. Binding was carried out at ambient temperature for 4 h with gentle agitation, after which the wells were washed four times with 10% FBS in PBS. The bound radioactivity was quantitated with a γ scintillation counter (LKB model 1277). Binding data was analyzed using a four-parameter fitting program (Kaleidagraph, Adelbeck Software). The receptor binding studies were repeated in the presence of heparin (10 μg/ml) to observe the maximal effect of heparin on the receptor-VEGF interaction. In these studies, we have used heparin as a commercial reagent to help address the biologic significance of the various forms of VEGF. Similar to the results observed by Tessler et al. (18) and Gitay-Goren et al. (26), dose-response studies with increasing heparin indicated the optimal concentration to achieve maximal KDR receptor binding.

Endocardial Cell Proliferation Assay for VEGF—Bovine adrenal cortical capillary endothelial cells were maintained in low glucose Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum, 2 mM glutamine, and antibiotics (growth medium) as described previously (1). For bioassay, cells were sparsely seeded in 12-well plates with 7 × 103 cells/well in 1 ml of growth medium. Samples of VEGF or VEGF variants (1 ml) were diluted in the culture media at concentrations from 1 μg/ml to 10 ng/ml (final) and layered onto the seeded cells. After 5 days, the cells were dissociated with trypsin and quantified using a Coulter Counter (Miami, FL).

RESULTS

Limited Proteolysis of VEGF—The susceptibility of VEGF to proteolytic digestion was explored using the glycosylated form of VEGF165, as expressed and purified from CHO cell conditioned medium. As observed in Fig. 2A (lane 2), VEGF165 appeared on SDS-PAGE as a doublet of proteins at 43 and 45 kDa. Tryptic mapping of reduced and carboxymethylated VEGF indicated approximately 75% of the protein contained N-linked glycosylation at Asn75, the remaining 25% of VEGF was unglycosylated (data not shown). The doublet of protein observed by non-reducing SDS-PAGE was due to partial glycosylation. As seen in other studies, VEGF165 from E. coli appeared as a single protein band of 38 kDa. A variety of enzyme digests of VEGF165 were prepared under similar conditions (1:100, enzyme/VEGF at 25 °C and for 20 h). Relative complete digestion was observed on reverse phase HPLC with broad-specificity enzymes, such as subtilisin, Pronase, proteinase K, and thermolysin. Enzymes such as plasmin, trypsin, chymotrypsin, and bromelain yielded varying extents of partial digestion resulting in a “core protein,” which was resistant to further proteolysis. No proteolysis was observed with thrombin, collagenase, elastase, S. aureus V8 protease, or pepsin as indicated by HPLC. The digestion profiles were similar for CHO- and E. coli-derived VEGF165. A series of enzyme digests were analyzed by non-reducing SDS-PAGE, which indicated that trypsin and plasmin cleaved VEGF, but thrombin, elastase, chymotrypsin, or pepsin did not (Fig. 2A). These enzyme digests were reduced, carboxymethylated, and evaluated by SDS-PAGE (Fig. 2B) to observe additional proteolysis that was not apparent under non-reducing conditions. Some proteolysis of VEGF by chymo-

**Fig. 2.** Enzymatic digestion of CHO-derived VEGF165. Digests with thrombin, plasmin, elastase, trypsin, chymotrypsin, and pepsin are evaluated in panel A, by non-reducing SDS-PAGE. In panel A, lanes 1, blank; lane 2, non-reduced CHO-derived VEGF165; lane 3, thrombin; lane 4, plasmin; lane 5, elastase; lane 6, trypsin; lane 7, chymotrypsin; lane 8, pepsin. In panel B, the samples were RCM after the enzyme digestion. Lane 1, non-reduced CHO-derived VEGF165; lane 2, RCM CHO-derived VEGF165; lane 3, thrombin; lane 4, plasmin; lane 5, elastase; lane 6, trypsin; lane 7, chymotrypsin; lane 8, pepsin digests of RCM CHO-derived VEGF165.
trypsin was observed (lane 7), in addition to the cleavage of VEGF by plasmin and trypsin (lanes 4 and 6). VEGF<sub>165</sub> is relatively resistant to proteolysis by enzymes such as collagenase, thrombin, and elastase. However, plasmin rapidly cleaves VEGF into discrete, non-disulfide-linked fragments.

Isolation and Identification of Plasmin-cleaved VEGF Fragments—To eliminate the apparent heterogeneity due to glycosylation of CHO cell expressed VEGF<sub>165</sub>, we studied the activity of plasmin on E. coli-derived VEGF<sub>165</sub>. The absence of carbohydrate on this form of VEGF was confirmed by tryptic mapping. A plasmin digest of VEGF<sub>165</sub> from E. coli (1:200 enzyme:substrate) was initiated and samples removed at various times for analysis by reverse phase HPLC. After 2 h of plasmin treatment, four protein peaks with absorption at 210 nm were resolved by HPLC (Fig. 3). The first peak, eluting at 12 min, exhibited minimal absorption at 280 nm, consistent with the carboxyl-terminal region of VEGF, which has only one aromatic amino acid, phenylalanine 128. Protein containing HPLC fractions were collected, pooled and identified by amino acid sequence, composition, and mass spectrometry. The early eluting peak of plasmin-cleaved VEGF<sub>165</sub> was identified as the polypeptide (111–165) by the amino-terminal sequence: Ala<sup>111</sup>-Arg-Gln-Glu-Asn-Pro<sup>116</sup>. The amino acid composition and minimal retention on reverse phase HPLC was consistent with the highly charged, hydrophilic 55-amino acid, carboxyl-terminal polypeptide. Mass spectral analysis indicated a molecular weight of 6473 atomic mass units for the (111–165) polypeptide (111–165), such that the heterodimer (1–165, 1–110) exhibited molecular weights of 38,306 and 25,385 atomic mass units, which compared well with the theoretical values of 38,300 and 25,389 atomic mass units, respectively. Retention times of these plasmin cleavage products are consistent with the loss of the hydrophilic carboxyl-terminal polypeptide (111–165), such that the heterodimer (1–165, 1–110) and homodimer (1–110) are progressively more hydrophobic and later eluting on reverse phase HPLC.

Kinetic Analysis of Plasmin Cleavage—Fig. 4 shows the kinetics for plasmin cleavage of E. coli-derived VEGF<sub>165</sub> (enzyme: substrate 1:100) at pH 7. The digest was analyzed at various times by reverse phase HPLC to quantify the amount of each form of VEGF. Cleavage of intact homodimer (1–165) occurred with a concomitant increase in the heterodimer (1–165, 1–110) followed by the appearance of the homodimer (1–110). The amount of (111–165) polypeptide increased over time and achieved a maximal level, which was approximately 40% of that observed for the (1–110) homodimer. Additional proteolysis of the cleaved polypeptide (111–165) by plasmin leads to lower molecular weight species, which eluted at 11 min on HPLC (Fig. 3). The plasmin catalyzed cleavage of VEGF<sub>165</sub> homodimer was approximately 3-fold faster than that observed for VEGF<sub>165/110</sub> cleavage, as indicated by the rate of disappearance for each protein observed on HPLC. The observation that VEGF<sub>165</sub> homodimer was cleaved faster than the VEGF<sub>165/110</sub> heterodimer was unexpected, since the two plasmin cleavage sites (Arg<sup>110</sup>-Ala<sup>111</sup> in each monomer) are identical in the dimer. We considered that the lysine-rich polypeptide (111–165) of VEGF may act as an additional recognition site for plasmin on VEGF and that the kringle domains (kringle 1, 4, and 5) may function as lysine-binding modules for blocking VEGF. Fig. 1 shows schematically the sequential cleavage of VEGF by plasmin. In the first cleavage, the intact carboxyl-terminal region of VEGF presented by the opposing VEGF<sub>165</sub> monomer may increase binding and cleavage of the full-length monomer, but does not participate in the binding of the heterodimer resulting in reduced rate for the second cleavage of VEGF<sub>165/110</sub>.

Heparin Binding of Homo- and Heterodimeric VEGF—The heparin binding function of VEGF<sub>165</sub> was studied by analytical affinity chromatography. Samples of E. coli-derived VEGF<sub>165</sub>, before and after treatment with plasmin, were applied to a POROS heparin column and eluted with an increasing gradient of NaCl. The eluate was monitored for optical density at 210 nm, and appropriate pooled fractions were analyzed by reverse phase HPLC. The results, shown in Table I, indicate that the heparin binding function of VEGF<sub>165</sub> is completely mediated by the carboxyl-terminal domain (111–165). The heparin affinity of VEGF<sub>165</sub> and the polypeptide (111–165) are nearly equivalent as determined by the concentration of NaCl required for elution (680 and 690 mM, respectively). Heterodimeric
VEGF165/110 eluted at 420 mM NaCl, indicating a significant decrease in heparin affinity associated with the loss of one carboxyl-terminal domain. No heparin binding was observed for homodimeric VEGF110 and VEGF121. Various conditions for plasmin digestion were used to prepare hetero- and homodimeric VEGF, followed by preparative heparin chromatography. Fig. 5 shows E. coli VEGF165, lacking carbohydrate modification, which yields a single band on SDS-PAGE with an apparent molecular mass of 38 kDa (lane 1). The SDS-PAGE analysis of a plasmin digest of E. coli VEGF165 appears in lane 2. VEGF110 homodimer was not retained on heparin-agarose (lane 3), while the (111–165) polypeptide with trace amounts of homo- and heterodimeric VEGF were eluted with 1 M NaCl (lane 4). Gradient elution of partial plasmin digests on a POROS-heparin column yielded highly purified preparations of VEGF165/110 and VEGF110 as indicated by reverse phase HPLC (data not shown).

Binding of VEGF Isoforms to Soluble Receptors in the Presence and Absence of Heparin—Binding of radioiodinated CHO-derived VEGF165 to KDR-IgG and FLT1-IgG was analyzed using a competitive displacement radioreceptor assay (Figs. 6 and 7). Various concentrations of unglycosylated (expressed in E. coli) VEGF165, VEGF165/110, VEGF110, and VEGF121 were tested for displacement of labeled glycosylated (expressed in CHO cells) VEGF165. The 55-amino acid, carboxyl-terminal domain of VEGF (111–165) was tested at 1000-fold molar excess and did not inhibit VEGF binding to the soluble receptors. At concentrations greater than 1000-fold molar excess, the isolated carboxyl-terminal domain appeared to partially displace VEGF165 from receptor. Radiolabeled VEGF binding to the soluble form of KDR was half-maximally displaced at 38 pM VEGF165 in the absence of heparin (Fig. 6A). Glycosylation of VEGF165 did not affect KDR binding, as indicated by the similar affinity exhibited by CHO- and E. coli-derived VEGF165 (Table II). Loss of one or both carboxyl-terminal domain(s) had no effect on the affinity of VEGF for KDR as indicated by the IC50 values for VEGF165/110 heterodimer and VEGF110 and VEGF121 homodimers (38, 29, and 30 pM, respectively). In the presence of heparin, the amount of VEGF bound to KDR was increased more than 3-fold; however, the apparent affinity of VEGF for KDR was unchanged (Fig. 6B). Glycosylated and unglycosylated VEGF165 bound KDR with equivalent IC50 values (31 and 28 pM, respectively). Loss of carboxyl-terminal domains resulted in 2-fold decreased KDR affinity (in the presence of heparin), as observed in competition studies with VEGF165/110 heterodimer and VEGF110 and VEGF121 homodimers (IC50 values of 45, 60, and 63 pM, respectively).

Glycosylated and unglycosylated VEGF165 exhibited similar affinity for soluble FLT-1 receptor, approximately 10 pM in the absence of heparin (Fig. 7A). Loss of one carboxyl-terminal domain was associated with 3-fold decreased FLT-1 affinity as indicated by the binding of VEGF165/110 heterodimer. The loss of both carboxyl-terminal domains resulted in approximately 10-fold reduced FLT-1 affinity as exhibited by the VEGF110 homodimer. Similarly, the values obtained for the natural splice variant VEGF121 indicated greater than 20-fold decreased binding to FLT-1 compared with that observed for VEGF165 (Table II). The carboxyl-terminal domain itself
(polypeptide 111–165) exhibited no binding to FLT-1 (as was observed with KDR). In the presence of heparin, the differences in FLT-1 binding observed for the VEGF variants were diminished (Fig. 7B). Glycosylated and unglycosylated VEGF 165 bound soluble FLT-1 with values for IC50 of 15 and 19 pm, respectively. VEGF 165/110 also exhibited similar binding affinity. The binding values for VEGF 121 and VEGF 110 were reduced approximately 2–3-fold, respectively, compared to those observed for VEGF 165.

Differential Stimulation of Endothelial Cell Growth Induced by VEGF Isoforms—Stimulation of endothelial cell proliferation by VEGF variants was evaluated with bovine adrenal cortical capillary endothelial cells (Fig. 8). The effective concentration to induce 50% of maximal stimulation (EC50) was determined in vitro by incubation of endothelial cells with VEGF or VEGF variants at concentrations that varied from 0.3 pm to 40 pm. Glycosylated and unglycosylated VEGF 165 exhibited similar IC50 values of 5.8 and 5.2 pm, respectively. The loss of one carboxyl-terminal domain resulted in approximately 7-fold decrease in potency for VEGF 165/110 as indicated by the higher EC50 value of 40 pm. Lower molecular weight variants, VEGF 110 and VEGF 121, displayed greater than 100-fold reduced potency with values of 2.58 and 2.56 nm for EC50, respectively. The isolated carboxyl-terminal domain (111–165) had no stimulatory effect on endothelial cell proliferation even with 4 orders of magnitude molar excess of polypeptide compared to the half-maximally effective concentration for VEGF 165. Similar results were observed with fetal bovine aortic endothelial cells with respect to a 10-fold and 100-fold loss in mitogenic potency with VEGF 165/110 and VEGF 110 (or VEGF 121), respectively (data not shown). These results demonstrate the critical role of the 111–165 region of VEGF in the stimulation of both large and small vessel endothelial cell proliferation.

**DISCUSSION**

Extracellular proteolysis and remodeling of the extracellular matrix play key roles in a variety of developmental processes (19). In addition, plasminogen activation and generation of plasmin have been shown to be important for the angiogenesis cascade (20). Such processes also play a major role in the local invasiveness and metastasis of tumor cells. Strong experimental evidence supports the concept that growth factors stored in the extracellular matrix and released in the course of its degradation are major mediators of such inductive processes. Numerous growth factors including fibroblast growth factor, platelet-derived growth factor, granulocyte/macrophage colony-stimulating factor, transforming growth factor-β, and leukemia inhibitory factor have been shown to be associated with the extracellular matrix (21–25).

Alternatively spliced molecular species of VEGF are differentially localized to heparan sulfate containing proteoglycans of the extracellular matrix or released as diffusible proteins (10, 11). In addition to this transcriptionally regulated heterogeneity, there appears the potential for proteolytic processing which may further regulate the bioavailability of VEGF. In the present study, we have demonstrated that plasmin readily cleaves VEGF 165 to yield a non-heparin binding isoform, VEGF 110, and a heparin-binding fragment composed of the carboxyl-terminal domain (111–165). In contrast to plasmin, neither thrombin, elastase, nor collagenase efficiently cleaves VEGF. Like VEGF 121, VEGF 110 has no affinity for heparin and is a diffusible molecule (11). Therefore, the carboxyl-terminal domain (amino acids 111–165) is completely responsible for the observed heparin binding of VEGF in vitro. Interestingly, a partially cleaved form, VEGF 165/110, was isolated that exhibited strongly reduced potency with VEGF 165/110 and VEGF 110 (or VEGF 121), respectively (data not shown). These results demonstrate the critical role of the 111–165 region of VEGF in the stimulation of both large and small vessel endothelial cell proliferation.
also reported increased binding of VEGF165 to Flk-1/KDR and did not significantly alter the dissociation constant. Tessler et al. (26) observed an increased activity of KDR for binding VEGF. The heparin-induced potentiation of VEGF binding to KDR has also been observed by other investigators. Gitay-Goren et al. (27) have observed the inhibition of heparin on VEGF binding to FLT-1 receptors on human melanoma cells. These investigators observed significantly decreased melanoma cell binding of 125I-labeled VEGF165 and VEGF105 in the presence of exogenous heparin (1 μg/ml) compared to that observed in the absence of heparin.

VEGF interaction with cell surface heparan-sulfate containing proteoglycans on endothelial cell growth has been examined by numerous investigators (18, 25–30). Sasisekharan et al. (30) have indicated a role for heparin-like molecules by inhibiting endothelial cell proliferation and in vivo neovascularization with heparinase. The binding of 125I-labeled VEGF165 to endothelial cells was completely inhibited by pretreatment with heparinase and could be restored by the addition of exogenous heparin (25). To evaluate the significance of the relative heparin affinity of VEGF isoforms, we tested the mitogenic activity of the 165, 165/110, 110, and 121 forms of VEGF on primary cultured bovine adrenal cortical capillary cells and fetal bovine aortic endothelial cells. Loss of one or both carboxy-terminal domains of VEGF significantly reduced the proliferation of endothelial cells. The mitogenic potency of VEGF165 and VEGF121 was substantially decreased (>100-fold) compared to VEGF165. VEGF165/110 exhibited 7–10-fold reduced activity on endothelial cell growth. These results are most interesting in light of the similarities and differences observed with various VEGF isoforms binding soluble KDR or FLT-1 receptors. The modestly decreased affinity observed with soluble KDR and FLT-1 receptors and the 110 and 121 isoforms of VEGF does not account for the drastically decreased endothelial cell mitogenic potency. This discrepancy suggests that the stability of VEGF-heparan-sulfate receptor complexes probably contributes to effective signal transduction and stimulation of endothelial cell proliferation. Further studies are clearly warranted to explore the regulatory function of heparin-like molecules in VEGF receptor interaction, signal transduction, and mitogenesis of endothelial cells.

Our findings indicate that VEGF, by alternative splicing and/or limited proteolysis, has the potential to express structural and functional heterogeneity that yields a graded biological response. Our current understanding of VEGF biology suggests the following sequence of events may occur during angiogenesis. Initially, cells respond to hypoxia or other stimuli by inducing VEGF transcription (31), resulting in increased expression of long and short forms of VEGF, although VEGF165 is probably the most abundant isoform. Compared to the longer forms of VEGF, the diffusible forms would migrate a greater distance, bind VEGF receptors, and trigger endothelial cell proliferation and migration. The intensity of the angiogenic signal would be weakest at the most distant sites, given the lesser mitogenic potency of VEGF110 and VEGF121. Closer to the site of VEGF synthesis (i.e. ischemic tissues), the concentration of the 165 isoform is expected to be increased due to extracellular matrix binding and the effect of the biochemical gradient would be enhanced with the associated greater mitogenic potency of VEGF165. In the most ischemic areas, matrix-associated VEGF is localized to the cells of origin with the highest concentration and potency. The heterogeneity of VEGF structure and function allows the formation of a biochemical gradient for the migration and chemotaxis of proliferating endothelial cells. In circumstances where plasminogen activation occurs (e.g. tumors and wounds), the presence of plasmin may serve to release stored forms of matrix-bound VEGF to amplify the angiogenic signal.

Placental growth factor (PIGF) is a protein with 53% homology to VEGF (32, 33). PIGF binds FLT-1 with similar affinity as VEGF, but does not bind to the KDR receptor (12). Interestingly, recent studies describing the existence of VEGF-PIGF heterodimers further extend the concept that structural heter-
oxygen may be responsible for a graded biological response. While the PI GF homodimer had minimal endothelial cell mitogenic activity compared to that of the VEGF homodimer, the heterodimer displayed intermediate activity (34). With respect to proteolytic digestion resulting in the generation of VEGF110 from VEGF165 or VEGF189, little is known at this time about the prevalence of this process in vivo. Nevertheless, the potential exists for VEGF to set up a biochemical gradient that radiates from an ischemic zone and may provide a directional signal for in vivo angiogenesis.

Acknowledgments—We thank William Henzel and Reed Harris for protein microsequencing, Allan Padua and Rodney Keck for amino acid analysis, Rex Hayes for purification of CHO- and E. coli-derived forms of VEGF165, and Lavon Riddle for purification of KDR and FLT-1 receptor-lgG chimeric protein.

REFERENCES

1. Ferrara, N., and Henzel, W. J. (1989) Biochem. Biophys. Res. Commun. 161, 851–858
2. Ploiet, J., Schilling, J., and Gospodarowicz, D. (1989) EMBO J. 8, 3801–3807
3. Ferrara, N., Houck, K., Jakeman, L., and Leung, D. W. (1992) Endocrinology 127, 965–968
4. Phillips, H. S., Hains, J., and Ferrara, N. (1990) Endocrinology 116, 521–532
5. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993) Nature 362, 841–844
6. Leung, D. W., Cachianes, G., Kuang, W.-J., Goeddel, D. V., and Ferrara, N. (1989) Science 246, 1306–1309
7. Tischer, E., Mitchell, R., Hartmann, T., Silva, M., Gospodarowicz, D., Fiddes, J., and Abraham, J. (1991) J. Biol. Chem. 266, 11947–11954
8. Houck, K. A., Ferrara, N., Winer, J., Cachianes, G., Li, B., and Leung, D. W. (1991) Mol. Endocrinol. 5, 1806–1814
9. Houck, K. A., Leung, D. W., Rowland, A., Winer, J., and Ferrara, N. (1992) J. Biol. Chem. 267, 26031–26037
10. Park, J. E., Keller, G. A., and Ferrara, N. (1993) Mol. Biol. Cell 4, 1317–1326
11. Park, J. E., Chen, H., Winer, J., Houck, K. A., and Ferrara, N. (1994) J. Biol. Chem. 269, 25646–25654
12. Ferrara, N., Winer, J., Burton, T., Rowland, A., Siegel, M., Phillips, H. S., Terrell, T., Keller, G. A., and Levinson, A. D. (1993) J. Clin. Invest. 91, 160–170
13. Hunter, W. M., and Greenwood, F. C. (1962) Nature 194, 495–496
14. Crestfield, A. M., Moore, S., and Stein, W. H. (1963) J. Biol. Chem. 238, 622–627
15. Laemmli, U. K. (1970) Nature 227, 680–685
16. Oakley, B. R., Kirsch, D. R., and Morris, N. R. (1980) Anal. Biochem. 105, 361–3635
17. Tessler, S., Rockwell, P., Hicklin, D., Cohen, T., Levi, B.-Z., Witte, L., Leminischa, I. R., and Neufeld, G. (1994) J. Biol. Chem. 269, 12456–12461
18. Matrisian, L. M., and Hogan, B. L. (1990) Curr. Top. Dev. Biol. 24, 219–259
19. Migliaccio, P., Tsuboi, R., Robbins, E., and Rifkin, D. B. (1989) J. Cell Biol. 108, 671–682
20. Vlodavsky, I., Folkman, J., Sullivant, R., Fridman, R., Ishai-Michaeli, R., Sass, J., and Klagesbrun, M. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 2292–2296
21. Raines, E. W., and Ross, R. (1992) J. Cell Biol. 116, 533–543
22. Gordon, M. Y., Riley, G. P., Watt, S. M., and Greaves, M. F. (1987) Nature 326, 403–405
23. Andres, J. L., Stanley, K., Cheifetz, S., and Massague, J. (1989) J. Biol. Chem. 264, 25646–25654
24. Maglione, D., Guerriero, V., Vigliette, G., Ferrara, M. G., Aprelikova, O., and Massague, J. (1991) J. Biol. Chem. 266, 12456–12461
25. Rathjen, P. D., Toth, S., Willis, A., Heath, J. K., and Smith, A. G. (1990) J. Biol. Chem. 265, 6093–6098
26. Gitay-Goren, H., Soker, S., Vlodavsky, I., and Neufeld, G. (1992) J. Biol. Chem. 267, 6093–6098
27. Cohen, T., Gitay-Goren, H., Sharon, R., Shibuya, M., Halaban, R., Levi, B.-Z., and Neufeld, G. (1995) J. Biol. Chem. 270, 11322–11326
28. Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M., and Heldin, C.-H. (1994) J. Biol. Chem. 269, 26980–26985
29. Soker, S., Goldstaudb, D., Svanh, C. M., Vlodavsky, I., Levi, B.-Z., and Neufeld, G. (1994) Biochem. Biophys. Res. Commun. 203, 1339–1347
30. Sasisekharan, R., Moses, M. M., Nugent, M. A., Cooney, C. L., and Langer, R. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 1524–1528
31. Levy, A. P., Levy, N. S., Wegner, S., and Goldberg, M. A. (1995) J. Biol. Chem. 270, 13333–13340
32. Maglione, D., Guarriero, V., Vigliette, G., Delli-Bovi, P., and Persico, M. G. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9267–9271
33. Maglione, D., Guarriero, V., Vigliette, G., Ferrara, M. G., Aprilello, O., Altaliko, K., Del Vecchio, S., Lei, K. J., Chou, J. C., and Persico, M. G. (1993) Oncogene 8, 925–931
34. DiSalvo, J., Bayne, M. L., Conn, G., Kwok, P. W., Trivedi, P. G., Soderman, D. D., Palisi, T. M., Sullivant, K. A., and Thomas, K. A. (1995) J. Biol. Chem. 270, 7717–7723
35. Ofner, C., D'Arcy, A., Winkler, F. K., Eggimann, B., and Hosang, M. (1992) EMBO J. 11, 3921–3926
The Carboxyl-terminal Domain(111165) of Vascular Endothelial Growth Factor Is Critical for Its Mitogenic Potency

Bruce A. Keyt, Lea T. Berleau, Hung V. Nguyen, Helen Chen, Henry Heinsohn, Richard Vandlen and Napoleone Ferrara

J. Biol. Chem. 1996, 271:7788-7795.
doi: 10.1074/jbc.271.13.7788

Access the most updated version of this article at http://www.jbc.org/content/271/13/7788

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 35 references, 19 of which can be accessed free at http://www.jbc.org/content/271/13/7788.full.html#ref-list-1