Neutrophil elastase cleaves VEGF to generate a VEGF fragment with altered activity

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Kurtagic E, Jedrychowski MP, Nugent MA. Neutrophil elastase cleaves VEGF to generate a VEGF fragment with altered activity. Am J Physiol Lung Cell Mol Physiol 296: L534–L546, 2009. First published January 9, 2009; doi:10.1152/ajplung.90505.2008.—Excessive neutrophil elastase (NE) activity and altered vascular endothelial growth factor (VEGF) signaling have independently been implicated in the development and progression of pulmonary emphysema. In the present study, we investigated the potential link between NE and VEGF. We noted that VEGF165 is a substrate for NE. Digestion of purified VEGF165 with NE generated a partially degraded disulfide-linked fragment of VEGF. Mass spectrometric analysis revealed that NE likely cleaves VEGF165 at both the NH2 and COOH termini to produce VEGF fragment chains ~5 kDa reduced in size. NE treatment of VEGF-laden endothelial cell cultures and smooth muscle cells endogenously expressing VEGF generated VEGF fragments similar to those observed with purified VEGF165. NE-generated VEGF fragment showed significantly reduced binding to VEGF receptor 2 and heparin yet retained the ability to bind to VEGF receptor 1. Interestingly, VEGF fragment showed altered signaling in pulmonary artery endothelial cells compared with intact VEGF165. Specifically, treatment with VEGF fragment did not activate extracellular-regulated kinases 1 and 2 (ERK1/2), yet resulted in enhanced activation of protein kinase B (Akt). Treatment of monocyte/macrophage RAW 264.7 cells with VEGF fragment, on the other hand, led to both Akt and ERK1/2 activation, increased VEGFR1 expression, and stimulated chemotaxis. These findings suggest that the tissue response to NE-mediated injury might involve the generation of diffusible VEGF fragments that stimulate inflammatory cell recruitment and activation via VEGF receptor 1.

PULMONARY EMPHYSEMA is defined by airspace enlargement as a result of alveolar septal cell death. Seminal findings, first reported in the 1960s, identified a strong link between emphysema and a deficiency in α1-antiprotease, leading to the elastase-antielastase imbalance hypothesis implicating neutrophil-released elastase as an important component to emphysema progression (36, 37, 79). However, it has become clear that the pathogenesis of emphysema is extremely complex and involves a wide range of biochemical and biomechanical components. Recent information suggests a convergence, to a certain degree, that identifies the inability of the lung to respond sufficiently to various forms of injury as a hallmark of disease (6, 68). In particular, excessive damage and lack of repair of the lung extracellular matrix (ECM) appear to play a central role in the ultimate loss of pulmonary function. In this light, we have focused on how injury to the pulmonary ECM alters its ability to control the access and response of cells to growth factors (7, 11, 12, 18, 65). In the present study, we report an interesting connection between neutrophil elastase (NE) and vascular endothelial growth factor (VEGF) with implications for lung injury and repair.

NE is a potent protease capable of degrading key components of connective tissue, including elastin (36, 37, 42, 70). Under conditions of tissue injury, elastase and an array of peptides, proteins, and other enzymes are released in the ECM by polymorphonuclear neutrophils. According to the protease-antiprotease hypothesis, for normal repair to occur, the subsequent activity of elastase is balanced by an antielastase screen of endogenous inhibitors, the predominant one being α1-antiprotease. However, under circumstances of overwhelming stimuli, oxidative inactivation, or genetic deficiency, the natural balance is disrupted, and a situation develops for elastase to evade local inhibitors, contributing to tissue destruction and the development of emphysema. NE is certainly not acting alone, since it has become clear that a large number of enzymes participate in lung injury. In particular, the large family of matrix metalloproteinases (MMPs) has been implicated in many aspects of lung pathology. Indeed, animals deficient in macrophage elastase (MMP12) are less susceptible to cigarette smoke-induced emphysema, and activation of MMP9 and MMP12 by interleukin-13 overexpression induces airspace enlargement (32, 82). NE has also been directly implicated in this process, since mice deficient in this protease show ~40% as much airspace enlargement as wild-type counterparts in response to cigarette smoke (67). Thus there is considerable need to better understand the consequences of NE injury within the pulmonary system.

VEGF is a critical factor involved in the development, growth, and survival of blood vessels (20, 66). Although the major tyrosine kinase receptors VEGFR1 (Flt-1) and VEGFR2 (KDR, flk-1) are expressed on vascular endothelial cells, there is also considerable evidence of wide expression of VEGFR1 within pulmonary epithelial cells, monocytes, and macrophages (23, 24). VEGF stimulates endothelial cell proliferation, migration, survival, and vascular permeability, with most of these activities being attributed to signaling through VEGFR2 and its tyrosine kinase activity. VEGFR1, on the other hand, is a kinase-impaired receptor whose role in mediating VEGF activity remains to be fully delineated (61). Targeted deletion of VEGFR1 resulted in early embryonic lethality because of excessive and unregulated blood vessel

1-antiprotease, leading to the elas-

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growth, suggesting that this receptor mediates a negative growth/survival signal in endothelial cells (25, 26, 43). Although the role of VEGFR1 signaling in endothelial cells remains controversial, recent data indicate that the nature of VEGFR1 signaling may vary depending on the cell type where it is expressed. For instance, VEGFR1 signaling in nonendothelial cells such as monocytes and trophoblasts stimulates cell migration and proliferation (3, 5, 14, 19).

The lung is among the organs with the highest levels of VEGF (48). Indeed, angiogenesis is clearly critical to pulmonary development (78), and even partial loss of endothelial function can drastically impair alveolarization (17). Recent evidence also suggests that VEGF contributes a protective activity against injury and oxidative stress. In this regard, a series of studies have implicated VEGF in experimental and human emphysema. Pharmacological inhibition of VEGFR2 activity and lung-targeted inactivation of the VEGF gene result in an emphysematous phenotype in rats and mice, respectively (41, 73). In human patients with emphysema, the expression of VEGF is reduced, and apoptosis is evident when compared with normal patients (40). In a separate study, patients with emphysema showed decreased levels of VEGF protein in sputum (39). Moreover, VEGF blockade induced the expression of oxidative stress markers and apoptosis in rats, leading to emphysema that was prevented by a superoxide dismutase mimic, suggesting a link between VEGF and oxidative stress (76).

As a further link between VEGF and emphysema, a recent report shows that cigarette smoke exposure reduced VEGF and VEGFR2 levels in rats and that human patients with chronic obstructive pulmonary disease showed reduced VEGFR2 expression (49). Interestingly, lung-targeted overexpression of VEGF induced interleukin-13 and an asthma-like phenotype with inflammation associated with an increased number of infiltrating leukocytes, edema, and parenchymal and vascular remodeling (44). Overexpression of placental growth factor (PlGF), a VEGF-like protein that binds VEGFR1 but not VEGFR2, caused emphysema with enlarged airspaces, and reduction in the number of endothelial cells (75). This range of observations clearly implicates VEGF as a factor that is under strict control within the healthy lung, since both decreased and increased levels show the potential to contribute to disease. An interesting link between these new observations implicating VEGF as a component of emphysema and the classic elastase-antielastase hypothesis is the fact that VEGF is normally stored within the ECM where it binds to heparan sulfate proteoglycans (HSPGs) and fibronectin (59, 64, 77). Thus elastase injury to the lung ECM is likely to have an impact on the storage, release, and activity of VEGF. Although the above studies have contributed significant information regarding the consequences of too much or too little VEGF in the lung, there is little information on how VEGF interactions with the ECM might be involved in the normal and pathological injury response in the lung.

In the present study, we investigated the possible link between NE-mediated injury and the VEGF pathway. We evaluated the sensitivity of VEGF to NE digestion and noted that NE partially degrades VEGF to generate a VEGF fragment that shows significantly reduced VEGFR2 and heparin binding activity, yet retains VEGFR1 binding. Interestingly, the NE-generated VEGF fragment showed altered signaling in pulmonary artery endothelial cells and in RAW 264.7 macrophage/monocyte cells compared with intact VEGF. These findings suggest that the release of a VEGF fragment with altered activity from ECM may be an additional consequence of NE injury of pulmonary tissue.

**MATERIALS AND METHODS**

**Materials**

Human recombinant VEGF165, recombinant human tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF)-AA, Quantikine human and rat VEGF immunoassay enzyme-linked immunosorbent assays (ELISAs), recombinant human VEGFR2/Fc chimera and VEGFR1/Fc chimera were purchased from R&D systems (Minneapolis, MN). Fibroblast growth factor 2 (FGF2) was from Chiron (Sunnyvale, CA). 125I-Bolton Hunter reagent was obtained from PerkinElmer (Boston, MA). 125I-labeled VEGF165, -FGF2, -TNF-α, and -PDGF were prepared using a modified Bolton-Hunter procedure as previously described (29, 55). Heparin was from Neoparin (San Leonardo, CA). BSA was obtained from American Bioanalytical (Natick, MA). RPMI-1640 low glucose media was purchased from Lonza Walkersville (Walkersville, MD). DMEM, PBS, penicillin/streptomycin, L-glutamine, and HEPES buffer were obtained from Invitrogen (Carlsbad, CA). CASE Kit for protein kinase B (Akt) S473 was purchased from SuperArray (Frederick, MD). Calf serum (CS) and FBS were purchased from HyClone (Logan, Utah). Primary antibodies for phosphor-extracellular-regulated kinase 1/2 (ERK1/2), total ERK1/2, Akt, and phospho-Akt were obtained from Cell Signaling Technology (Danvers, MA). Anti-VEGF polyclonal antibodies raised to full-length VEGF (Upstate no. 06–565) and to a synthetic peptide encompassing an internal region conserved among all VEGF isoforms (Millipore no. 07-1376) were obtained from Millipore (Billerica, MA). Human neutrophil MMP9 and VEGFR kinase inhibitor III were purchased from Calbiochem (San Diego, CA). NE (human) and Porcine Pancreatic Elastase (PPE) were obtained from Elastin Products (Owensville, MO). 4-Aminophenylmercuric acetate was purchased from Sigma-Aldrich (St. Louis, MO).

**Cell Culture**

Bovine aortic endothelial cells (BAEC passage 5–15) were maintained in low-glucose DMEM, supplemented with 10% CS, 5 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Bovine pulmonary artery endothelial cells (bPAEC passage 5–10) were purchased from Cambrex Bio Science Walkersville and maintained in low-glucose DMEM, supplemented with 10% FBS, 5 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. Mouse macrophage/monocyte RAW 264.7 cells were propagated in RPMI-1640 with 5 mM L-glutamine, 10% heat-inactivated FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate (63). FBS was inactivated by heating for 30 min in a 56°C water bath, mixing every 10 min. Neonatal rat aortic smooth muscle cells (SMCs) were isolated from Sprague-Dawley rats, ages 1–3 days as described (22), and maintained in low-glucose DMEM, with 10% FBS, 5 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate and 1% nonessential amino acids. Neonatal rat lung fibroblasts were isolated and maintained in low-glucose DMEM, with 5% FBS, 5 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate and 1% nonessential amino acids (27).

**VEGF Fragment Generation**

125I-VEGF165 was treated with varying NE or PPE concentrations in 44 mM sodium bicarbonate, pH 7.4, for various times at 37°C. Mock-treated 125I-VEGF165 was incubated in 44 mM sodium bicarbonate, pH 7.4 at 37°C. The reaction was stopped by adding 1 μM di-isopropyl fluorophosphate (DFP), and samples were subjected to
15% SDS-PAGE under reducing and nonreducing conditions, followed by gel fixation and phosphor screen visualization of the bands. For large VEGF fragment (VEGFf) preparations, the samples (NE and mock treated) were dialyzed (10-kDa molecular weight cut off, Slide-A-Lyzer; Pierce, Rockford, IL) against PBS at 4°C to remove DFP. The concentration of 125I-VEGF165 and 125I-VEGFf in the samples was determined by trichloroacetic acid precipitation. A silver stain kit (Owl Scientific, Portsmouth, NH) was also used for visualization of the protein bands. Further purification of VEGFF was performed using heparin-Sepharose CL6B beads (GE Healthcare Bio-Science, Uppsala, Sweden). VEGFf at various NaCl concentrations in PBS was incubated with heparin-Sepharose beads (1:1 slurry) for 1 h at 4°C while rotating. Heparin-bound VEGFf was separated from the unbound VEGFf by centrifugation at 1,000 g for 3 min. Supernatants were analyzed by SDS-PAGE and phosphor screen visualization.

Mass Spectrometry

To evaluate the physical alterations of VEGF caused by NE digestion, we used carrier-free, purified recombinant VEGF165. Disulfide bonds in VEGF and VEGFf treated with NE were reduced with dithiothreitol (10 mM, 100°C, 15 min), and all cysteine residues were blocked by treatment with iodoacetamide in the dark (100 mM, 57°C, 30 min). Full-length and eluate-treated VEGF were subjected to 12% SDS-PAGE followed by Coomassie blue staining. The Coomassie-stained VEGF bands were excised, cut further to 1-mm cubes, and subjected to in-gel trypsin digestion as previously described (46). Extracted peptides were dried to completion by vacuum centrifugation.

Liquid chromatography-mass spectrometry/mass spectrometry data were obtained using a LTQ Orbitrap (ThermoFisher, San Jose, CA) mass spectrometer. Dried peptides were resuspended in 10 µl of 5% acetonitrile/3% acetic acid, and 4 µl were loaded on a pulled fused silica microcapillary column (125 µm ID, 12-cm bed) packed with C18 reverse-phase resin (Magic C18AQ, 5 µm particles; 200 Å pore size; Michrom Bioresources, Auburn, CA). Peptides were resolved using an Agilent 1100 series binary pump across a 30-min linear gradient of 8–25% acetonitrile in 0.2% formic acid at a 250 µl/min flow rate. In each data collection cycle, one full mass spectrometry (MS) scan (375:1,600 mass-to-charge ratio) was acquired in the Orbitrap (6 × 10^9 resolution setting; automatic gain control target of 106) followed by 10 data-dependent MS/MS scans in the LTQ (AGC target 5,000; threshold 3,000) using the 10 most abundant ions for collision-induced dissociation for fragmentation. The method dynamically excluded previously selected ions for 30 s, singly charged ions, and unassigned charged states.

Raw files obtained from the data collection were converted into mzXML format using the ReAdW program (http://sashimi.sourceforge.net/software_glossolalia.html). Monoisotopic precursor ion and charge state information for each acquired MS/MS spectra were extracted by in-house software. SEQUEST search algorithm was used to search the MS/MS spectra against the HUMAN.NCI database. The search parameters for posttranslational modifications included a static modification of 57.02146 Da on cysteine (carboxyamidomethylation) and dynamic modification of 15.99491 Da for methionine (oxidation) residues.

VEGF165 and VEGF165 Fragment Binding Assays

Binding to VEGFR1/2. Binding assays were performed with VEGFR chimeras by incubating a range of 125I-VEGF165 and 125I-VEGFf concentrations (0.05, 0.1, 0.25, 0.5, and 1 nM) with Fc-VEGFR1 or VEGFR2 (0.1 nM) in binding buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, and 1 mg/ml BSA) for 2 h at 4°C. The bound complexes were pulled down with magnetic protein-A beads (New England Biolabs, Beverly, MA). The beads were washed three times with binding buffer, and 125I-VEGF165/125I-VEGFf associated with the beads was measured using a Coba Auto-Gamma 5005 counter (Packard Instruments, Meridian, CT).

Binding to heparin. Ninety-six-well plates that have been functionalized by plasma polymerization to contain a controlled layer of amines (generously supplied by Plasgo Technology, Sheffield, UK) were used. Heparin (1 µg/ml in PBS) was complexed to the surface of the plate overnight at room temperature (RT). The plates were washed and then incubated with 125I-VEGF165 and 125I-VEGFf in binding buffer (0.15 M NaCl, 25 mM HEPES, pH 7.5) for 2 h at 4°C. Unbound VEGF/VEGFf was washed, and heparin-bound VEGF/VEGFf was extracted with 1 M NaCl, 25 mM HEPES (pH 7.5), and 0.5% Triton X-100. Radioactivity was counted using a Coba Auto-Gamma 5005 counter.

VEGF binding and release from endothelial cells. BAECs were plated at 5 × 10^4 cells/well for 24-well plate and grown to confluence. Cells were incubated with binding buffer [25 mM HEPES, pH 7.5 in DMEM (without bicarbonate) containing 0.1% BSA] for 10 min at 4°C to inhibit endocytosis and binding site turnover. 125I-VEGF and VEGFf were added to the cells and incubated for 2.5 h at 4°C. Unbound VEGF/VEGFf was washed away, and cells were incubated with 44 mM NaHCO3, pH 7.4, with and without PPE (0.5 µg/ml) for various times at RT. The quantity of 125I-VEGF165/125I-VEGFf in the PPE digest was determined by counting the radioactivity in a gamma counter. The state of the 125I-VEGF165 was evaluated by subjecting samples to 15% SDS-PAGE followed by phosphor image analysis. PPE was used in these experiments to avoid the complications of heparan sulfate-mediated inhibition of NE (71). The VEGF generated by PPE treatment of purified VEGF165 was indistinguishable to that generated by NE.

VEGFf Release from Smooth Muscle and Fibroblast Cell Cultures

Rat aortic SMCs and rat lung fibroblast cells were kept in culture for 4 and 3 wk, respectively. At the onset of the experiment, cultures were rinsed with 44 mM NaHCO3, pH 7.4. Cells were then incubated with 44 mM NaHCO3 with and without PPE or NE (5 µg/ml) for the indicated time (15 or 30 min) at 37°C. The NaHCO3 solutions (referred to as elastase digests) were collected, and elastase was inactivated with 1 µM DFP. Samples were centrifuged at 800 g for 10 min at 4°C, and the supernatants were concentrated using Amicon ultra centrifugal filter devices (10,000 MWCO; Millipore). The presence of VEGF and VEGFf was evaluated using VEGF ELISA. Samples were also subjected to 15% SDS-PAGE and analyzed by immunoblot to visualize VEGF and VEGFf released.

Cell Signaling

ERK1/2 and Akt activation were evaluated in response to VEGF and VEGFf, bPAEC and RAW 264.7 cells were plated at 10^4 cells/ml in six-well dishes. After 24 h, the cells were serum starved overnight in 0.5% serum to quiesce the cells. The cells were incubated in medium (DMEM, 25 mM HEPES, 1 mg/ml BSA) for 90 min followed by treatment with VEGF and VEGFf at 37°C for 10 min. After the incubation, the medium was removed, and the cells were extracted in lysis buffer (150 mM NaCl, 10 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM sodium vanadate). Lysates were centrifuged, and the supernatant was subjected to 12% SDS-PAGE and analyzed by immunoblot to measure phosphorylated (p) ERK1/2, total ERK1/2, pAkt, and total Akt. pAkt and total Akt were also measured using a cell-based CASE Kit. RAW and bPAEC cells were seeded in 96-well tissue culture-treated plates at 15,000 cells/well. Later (24 h), cells were serum starved overnight in serum-free media. VEGF or VEGFf were added directly to the cultured media (0.45 nM), and cells were incubated at 37°C for 10 min. After the incubation, the levels of pAkt and total Akt were measured according to the SuperArray Kit protocol.
Real-Time PCR

RAW 264.7 cells were grown to ~70% confluence, serum starved overnight in 0.5% FBS, and treated with 0.45 nM VEGF/VEGFf for 30 h. Total RNA was extracted from the cells in 4 M guanidinium thiocyanate as described previously (80). Genomic DNA was removed by incubation with RNase-free DNase I (M0303S; New England BioLabs) in the presence of RNase inhibitor. The RNA was annealed with oligo(dT) and random hexamer primers, and first-strand synthesis was carried out with MuLV reverse transcriptase. Negative controls were performed without reverse transcriptase. Real-time PCR was performed on ABI 7300 using ABI TaqMan gene expression assays as follows: VEGFR1 Mm00438980 and the Eukaryotic 18S rRNA Endogenous Control, 4308329. The cycling parameters were 50°C for 10 min, 95°C for 2 min, 45 cycles of 95°C for 15 s, and 60°C for 1 min. Results were calculated using the ΔΔCt method using 18S rRNA as the endogenous control.

Cell Migration

Wells in the upper chamber of a 96-well multiscreen-MIC plate (8 μm pore size; Millipore) were coated with 60.5 μg/ml type I collagen overnight at RT. The following day, RAW 264.7 cells (5,000 cells/well) were seeded in serum-free media without phenol red dye. The wells in the lower plate filled with serum-free media with or without chemotactrant and the upper plate containing cells were placed on top of the feeder plate and incubated for 4 h at 37°C. After the migration period, the upper plate containing cells was separated from the feeder plate; media were discarded, and the migrated cells on the outer side of the membrane were detached by incubating in TrypZean solution (Sigma-Aldrich) for 30 min at 37°C. Detachment solution containing migrated cells was transferred to an ultraviolet transparent 96-well plate, and the cells were stained and detected using the CyQUANT NF cell proliferation kit (Invitrogen). Fluorescence was read using excitation at 485 nm and emission at 530 nm using a plate reader (Tecan Infinite M200, San Jose, CA).

Statistical Analysis

Statistical significance of data included in this paper was evaluated using ANOVA followed by the Newman-KeUL’s multiple-comparison t-test. Differences were considered significant when P values were <0.05. Data are presented as the means of replicate samples ± SE or ± SD as indicated in the legends to Figs. 1–10.

RESULTS

Elastase Partially Degrades VEGF

Uncontrolled elastase activity is a hallmark of lung pathology. Recent studies have also revealed that disruption of VEGF-mediated endothelial cell survival can disrupt lung function and can lead to the development of an emphysematic phenotype. VEGF is normally stored in ECM, where it binds to HSPGs and fibronectin, and is essential for endothelial cell survival and repair in the peripheral lung. To explore the possible connection between elastase-mediated lung injury and VEGF, we assessed the sensitivity of VEGF165 to NE cleavage. 125I-VEGF165 was incubated with a range of NE concentrations for 30 min. We observed that VEGF165 was partially degraded by NE and that, after 30 min of incubation, most of the VEGF165 was cleaved to a lower-molecular-mass fragment (Fig. 1A). NE concentrations as low as 10 μg/ml led to significant VEGF digestion and production of a smaller VEGFf (Fig. 1B). Interestingly, the major VEGFf generated appeared to be significantly resistant to further digestion with NE since increasing NE concentrations up to 50 μg/ml did not show further degradation.

Active VEGF is a disulfide-linked dimer; hence, we assessed whether the NE-generated VEGFf remains disulfide-linked by comparing the migration of 125I-VEGF and 125I-VEGFf in SDS-PAGE under reducing and nonreducing conditions. The
major band of intact VEGF migrated with apparent molecular mass of ~44 kDa under nonreducing conditions, corresponding to a disulfide-linked VEGF dimer. Under reducing conditions, the major VEGF band showed a relative molecular mass of ~22 kDa. NE-digested VEGF showed a major band at ~17 kDa under reducing conditions. However, under nonreducing conditions, VEGFf showed a major band of ~34 kDa, indicating that the majority of VEGFf is also a disulfide-linked dimer (Fig. 1C).

To analyze the physical alterations in VEGF when treated with NE, we digested purified recombinant VEGF165 with NE, fully reduced and blocked all cysteine residues, and visualized the products using SDS-PAGE and silver staining. In the first lane in Fig. 2A, there is a band corresponding to intact mock-treated VEGF at ~22 kDa. Fully reduced and disulfide-blocked VEGFf formed after NE treatment produced a distribution of bands with the major species migrating to ~17 kDa. There was a clear distinction between VEGFf and NE (NE migrates to ~29 kDa).

VEGF and VEGFf were also analyzed by mass spectrometry. Liquid chromatography-MS/MS was performed, and the data were searched using SEQUEST set to a mass tolerance of 1.1 D. Both VEGF and VEGFf bands produced peptides that were identified as human VEGF165. Figure 2B shows mock-treated VEGF sequence coverage retrieved by mass spectrometry compared with sequences retrieved from VEGFf. Comparison of the two indicates that VEGF165 is likely cleaved by NE on the NH2 terminus and COOH terminus to generate a major fragment that is ~5 kDa smaller in size than intact VEGF165. There are likely also some cleavage sites within the protein that produce the smaller proteins observed when the protein is fully reduced (Fig. 2A).

**VEGFf is Released from VEGF-Laden Endothelial Cell Cultures**

Because we were able to observe VEGFf formation in vitro, we expanded our studies to tissue culture, where we used BAECs, which are known to bind and incorporate a considerable amount of VEGF in their ECM (29). 125I-VEGF165 was incubated with BAECs until binding reached equilibrium. Unbound VEGF was removed, and the VEGF that remained bound to the ECM and VEGFR was released by incubation in buffer in the presence and absence of PPE. We observed a rapid release of bound 125I-VEGF as early as 5 min after elastase treatment was initiated (Fig. 3A) with the total quantity of VEGF released being greater in the elastase condition compared with control. Interestingly, when we visualized the state of the released VEGF using SDS-PAGE and autoradiography, we noted the appearance of VEGFf along with intact VEGF in the elastase-treated cultures (Fig. 3B). Thus elastase was able to generate VEGFf by digesting VEGF-impregnated cell cultures.

To determine if elastase can release endogenously synthesized VEGF from cells, we treated lung fibroblast cultures with PPE (5 µg/ml, 15 min) or buffer alone and used a quantitative ELISA to measure VEGF and noted increased levels of VEGF released in the PPE-treated cells (3.2 ± 0.2 pg/ml in PPE...
digests compared with 0.0 pg/ml in control digests). Although the effect of PPE on lung fibroblasts was reproducible, the low levels of VEGF released prevented further analysis of the fragmented state of the elastase-released VEGF. Thus, to investigate the state of elastase-released endogenous VEGF, we used primary SMCs that produce high levels of VEGF when maintained in culture for prolonged periods. SMCs were cultured for 4 wk to allow for the accumulation of VEGF within the ECM and then subjected to PPE treatment (5 μg/ml; 30 min) and VEGF ELISA. PPE treatment resulted in release of VEGF (61.2 ± 14.0 pg/ml in the PPE digests compared with 6.4 ± 1.6 pg/ml in the control digests).

These samples and similar ones from NE-treated cells were subjected to SDS-PAGE and Western blot analysis with two separate VEGF antibodies. Control and elastase digests showed a number of anti-VEGF immunoreactive bands in the molecular mass range consistent with the presence of the various VEGF isoforms. However, lower-molecular-mass bands were observed in the PPE and NE digests that were not present in the untreated samples. There were also some interesting differences in the bands recognized by the two VEGF antibodies. There were VEGFf bands similar to those observed with human recombinant VEGF<sub>165</sub> in the blots analyzed with the polyclonal antibody raised to full-length VEGF (Fig. 4, A and C), whereas there were also smaller-fragment bands observed with the antibody raised to the internal region of VEGF (Fig. 4, B and D). Although the exact epitopes recognized by these antibodies are not known, it is possible that the various bands represent fragments of particular VEGF isoforms that are selectively recognized by the two antibodies used. Importantly, these data indicate that endogenous VEGFf appear to be generated when VEGF-rich cell cultures are subjected to elastase treatment.

**Specificity of VEGFf Formation**

To evaluate the specificity of elastase generation of VEGFf, we subjected other growth factors (FGF<sub>2</sub>, PDGF) and cytokines (TNF-α) to NE digestion (Fig. 5A). We observed that PDGF was also cleaved to generate a smaller fragment. This is an interesting finding, given that PDGF is a structural homolog of VEGF, characterized by highly conserved cysteine residues making a cysteine knot motif (53). Interestingly, TNF-α did not appear to be cleaved to a smaller elastase-resistant fragment; instead, the band intensity of intact TNF-α was reduced compared with the control, indicating that TNF-α is more fully

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**Fig. 3.** 125I-VEGF<sub>165</sub> binding and release from endothelial cells. A: confluent cultures of bovine aortic endothelial cells were incubated with 0.23 nM 125I-VEGF<sub>165</sub> at 4°C for 2 h, and unbound 125I-VEGF<sub>165</sub> was removed by washing the cells three times in binding buffer. The 125I-VEGF<sub>165</sub>-bound cells were incubated in 44 mM sodium bicarbonate buffer, pH 7.4, ±0.5 μg/ml porcine pancreatic elastase (PPE) for the indicated time. Released 125I-VEGF<sub>165</sub> was counted using a γ-counter. ○, 125I-VEGF released with PPE; ○, 125I-VEGF released in bicarbonate buffer. Each data point represents the mean of triplicate determinations ± SD. Statistical analysis revealed a significant difference between PPE and buffer-treated cells (p < 0.01). The experiment was repeated more than three times with similar results. B: 44 mM sodium bicarbonate buffer, pH 7.4, containing released 125I-VEGF<sub>165</sub> was collected from the cells from three separate wells after the indicated incubation period and was subjected to 15% SDS-PAGE and PhosphorImager analysis.

**Fig. 4.** Endogenous VEGF and VEGF fragments are released from smooth muscle cells (SMCs). SMCs were maintained in culture for 4 wk and then subjected to treatment with 44 mM NaHCO<sub>3</sub> with or without 5 μg/ml PPE (A and B) or 5 μg/ml NE (C and D) for 30 min at 37°C. Elastase digests were collected, and 1 μM was DFP added. Digests were centrifuged (800 g, 10 min at 4°C) and concentrated (10,000 MWCO centrifugal devices). PPE (A and B) and NE (C and D) digest samples were subjected to 15% SDS-PAGE and analyzed by immunoblot with anti-VEGF polyclonal antibody raised to full-length VEGF<sub>165</sub> (no. 06–565; A and C) or anti-VEGF polyclonal antibody raised to the internal region of VEGF (no. 07-1376; B and D).
digested by NE. FGF2 seems to be considerably resistant to NE cleavage, with NE-treated FGF2 being of similar intensity as intact FGF2.

To test whether other ECM proteases cleave VEGF to produce partially digested fragments, we treated VEGF with MMP9, since MMP9 has also been implicated in pulmonary matrix destruction and the development of emphysema. Even with prolonged incubations of $^{125}$I-VEGF$_{165}$ with MMP9, little VEGF degradation was detected (Fig. 5B). Under these same conditions, these MMP9 samples (10, 2.5, and 1 µg/ml) effectively solubilized 1 mg/ml of its known target protein Gelatin type B (data not shown). Hence, the cleavage of VEGF$_{165}$ by NE does not appear to reflect a general property of all extracellular proteases.

**VEGFf Shows Altered Heparin and Receptor Binding**

To investigate the activity of the elastase-generated VEGFf, we prepared large batches of VEGFf and mock-treated VEGF for comparison. PhosphorImager analysis revealed >98% reduction in the full-length VEGF by NE (Fig. 6A). Assuming that elastase modulates VEGF upon lung injury, which would lead to altered ability of VEGF to bind to HSPG sites within the ECM, we performed experiments to assess VEGFf binding to heparin. Ninety-six-well plates containing an amine-coated surface were complexed with heparin, and VEGF/VEGFf binding was examined. The results indicate that VEGFf showed reduced ability to bind to heparin compared with intact VEGF (Fig. 6B). This is consistent with mass spectrometry data that indicate that VEGF is cleaved on the COOH terminus, which contains the major heparin-binding domain. Knowing that VEGFf has a much lower affinity for heparin binding than intact VEGF, we used this characteristic to further purify VEGFf from the intact VEGF in the master preparation. To do so, we incubated VEGFf with heparin-Sepharose beads with increasing salt concentrations. Proteins bound to heparin-Sepharose beads were separated from the unbound fractions by centrifugation, and the unbound solution was examined by SDS-PAGE and PhosphorImager analysis. At very low salt concentration, such as 0.15 M, most of the intact VEGF and VEGFf remained bound to heparin; thus, we see a very light band representing VEGFf that had not bound to the heparin-Sepharose beads. When the salt concentration was increased to 0.25 M, intact VEGF still remained bound to heparin, whereas the majority of the VEGFf was dissociated from heparin and released in the supernatant. Only at the highest salt concentrations tested (0.5 M) did we begin to observe intact VEGF dissociation from heparin. Therefore, we used heparin-Sepharose with 0.25 M NaCl to purify VEGFf from the small amount of intact VEGF present in the NE-treated VEGF preparations.

We proceeded to determine whether VEGFf retained the ability to bind to its major tyrosine kinase receptors VEGFR1 and VEGFR2 using VEGFR-Fc chimeras. Intact VEGF was pulled down with VEGFR1-Fc and VEGFR2-Fc chimeras, indicating its binding affinity for both receptors (Fig. 7A). However, VEGFf was pulled down complexed with VEGFR1-Fc only, and very little VEGFf was pulled down complexed to the VEGFR2-Fc chimera (Fig. 7B). The results indicate that VEGFf has retained the ability to bind to VEGFR1, whereas it has lost the ability to bind VEGFR2.

**Fig. 5. Evaluating the specificity of VEGF degradation. A: 50 ng/ml of $^{125}$I-VEGF$_{165}$, $^{125}$I-fibroblast growth factor 2 (FGF2), $^{125}$I-platelet-derived growth factor (PDGF), and $^{125}$I-tumor necrosis factor (TNF)-α were incubated with 20 µg/ml NE in 44 mM sodium bicarbonate buffer, pH 7.4, at 37°C for 30 min. The reaction was stopped by boiling in reducing SDS-PAGE sample buffer, and samples were subjected to 15% SDS-PAGE, followed by gel fixation and PhosphorImager analysis. B: $^{125}$I-VEGF$_{165}$ was treated with 10 µg/ml activated matrix metalloproteinase (MMP) 9 [activation by 4-aminophenylmercuric acetate (APMA) (72)] for the indicated times at 37°C, and the reaction was terminated by boiling in reducing SDS-PAGE sample buffer. Samples were subjected to 15% SDS-PAGE, followed by gel fixation and PhosphorImager analysis.**

![Image](http://ajplung.physiology.org/)
VEGF Activity is Altered by Elastase Cleavage

We tested VEGFf activity in endothelial cells by looking at the activation of ERK1/2, a major downstream target of VEGFR2 signaling (35). VEGFf compared with mock-treated VEGF did not show significant stimulation of ERK1/2. Interestingly, when VEGFf was added along with VEGF, there was an increase in ERK1/2 activation compared with that observed with VEGF alone (~2-fold, Fig. 8A). These data suggest that VEGFf might potentiate VEGF activity, potentially by binding to VEGFR1 and preventing intact VEGF binding, thus increasing the fraction of VEGF available to bind VEGFR2. We also investigated Akt activation upon VEGF and VEGFf stimulation and noted that Akt was activated by both intact and cleaved VEGF (Fig. 8B) with the response to VEGFf being slightly greater than that observed with intact VEGF. Cosimulation of endothelial cells with VEGF and VEGFf did not show increased Akt activation above that observed with VEGFf alone (data not shown). The Akt activation was confirmed using a cell-based ELISA as well where VEGFf showed twofold greater Akt activation than intact VEGF (Fig. 8C). These findings suggest that VEGFf binding to VEGFR1 can mediate Akt activation in these cells.

To further explore the biological effects mediated by VEGFf, the mouse macrophage/monocyte cell line RAW 264.7 was used. This cell line expresses only VEGFR1 and has no Akt activation upon VEGF and VEGFf stimulation and noted that Akt was activated by both intact and cleaved VEGF (Fig. 8B) with the response to VEGFf being slightly greater than that observed with intact VEGF. Cosimulation of endothelial cells with VEGF and VEGFf did not show increased Akt activation above that observed with VEGFf alone (data not shown). The Akt activation was confirmed using a cell-based ELISA as well where VEGFf showed twofold greater Akt activation than intact VEGF (Fig. 8C). These findings suggest that VEGFf binding to VEGFR1 can mediate Akt activation in these cells.

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We tested VEGFf activity in endothelial cells by looking at the activation of ERK1/2, a major downstream target of VEGFR2 signaling (35). VEGFf compared with mock-treated

Fig. 6. VEGF fragment (VEGFf) binding to heparin. A: a batch of VEGFf was prepared by incubating 125I-VEGF165 (33.3 μg/ml) with NE (20 μg/ml) in 44 mM sodium bicarbonate buffer, pH 7.4 for 30 min at 37°C. The reaction was stopped by adding 1 μM DFP, and the samples were dialyzed exhaustively (10 kDa MWCO) against PBS at 4°C. A fraction was analyzed by 15% SDS-PAGE followed by gel fixation and PhosphorImager visualization. B: 96-well plates were coated with 1 μg/ml heparin by overnight incubation in PBS. Plates were incubated with 125I-VEGF and 125I-VEGFf (0.11, 0.23, 0.68, and 1.14 nM) in binding buffer (0.15 M NaCl, 25 mM HEPES, pH 7.5) for 2 h at 4°C, and bound VEGF/VEGFf was extracted with 1 M NaCl, 25 mM HEPES (pH 7.5), and 0.5% Triton X-100. Samples were counted in a gamma counter. C: 125I-VEGF bound; , 125I-VEGF bound. Each data point is the mean of quadruplicate determinations ± SD. The binding of intact VEGF and VEGFf to heparin was significantly different (P < 0.01). Similar results were observed in three separate experiments.
detectable VEGFR2 expression. RT-PCR analysis confirmed the expression of VEGFR1 mRNA in these cells, whereas VEGFR2 mRNA was undetectable, even after 45 cycles (data not shown). When we treated RAW 264.7 cells with VEGF and VEGFf, we saw that both were similarly capable of activating ERK1/2 (Fig. 9A). This is in contrast to the response we observed with endothelial cells, where VEGF alone had no effect (Fig. 8A). To evaluate whether the ERK1/2 activation was mediated through VEGFR1, we preincubated cells with a known VEGFR kinase inhibitor (VEGFR kinase inhibitor III), before stimulation with VEGF or VEGFf. The VEGF and VEGFf effects on ERK1/2 activation were completely abolished in the presence of the VEGFR kinase inhibitor (Fig. 9A). To ensure that the effect of VEGFf is not complicated by the presence of DFP-inactivated NE in the VEGFf preparation, we treated cells with NE that had been inactivated with DFP and observed no ERK1/2 activation (data not shown). It is known that PI(3)K has strong homology to VEGF and binds and mediates its effects through VEGFR1 (14). Thus, we tested the effects of PI(3)K on RAW 264.7 cells and observed ERK1/2 activation, similar to that observed with VEGF or VEGFf (Fig. 9B).

We also evaluated Akt activation in RAW 264.7 cells in response to VEGFf treatment. Similar to our observations with endothelial cells, both VEGF and VEGFf activated Akt, and VEGFf appeared to be more effective (Fig. 9C). VEGF is known to stimulate the expression of VEGFR1 (4); thus, we tested the effects of VEGF and VEGFf on VEGFR1 mRNA levels. RAW 264.7 cells were treated with 0.45 nM VEGF or VEGFf for 30 h, and total RNA was isolated and analyzed by real-time PCR. Figure 9D shows that both VEGF and VEGFf treatment increase VEGFR1 mRNA levels and that VEGFf was more potent in doing so.

Because it had been reported that VEGF and PI(3)K are monocyte chemoattractants (14), we assessed whether VEGFf has any effect on monocyte migration. To do so, we added VEGF, VEGFf, PI(3)K, or TNF-α to the lower chamber of a migration plate and measured RAW 264.7 cell migration after 4 h. VEGF, VEGFf, and PI(3)K stimulated RAW cell migration to a similar extent, and TNF-α was somewhat more effective at the concentration tested. The addition of serum to the lower chamber enhanced RAW migration as well, as previously reported (57) (data not shown).

**DISCUSSION**

VEGF is a critical factor for normal vascular development and angiogenesis, with even single allele deletions being embryonic lethal between days 11 and 12 (13). In addition to its well-known role as an endothelial cell survival factor and mitogen, recent data have suggested important roles for VEGF in other cell types. For example, VEGF has been demonstrated to stimulate monocyte chemotaxis (5, 14), enhance colony formation of granulocyte-macrophage progenitors (9), and increase B cell production (31). VEGF has also been suggested to stimulate surfactant production by alveolar type II cells (15) and induce distal airway epithelial cell proliferation (8) in explant and whole lung studies; however, studies with isolated alveolar type II cells suggest that these effects are indirect and likely reflect paracrine interactions involving additional lung cell types (30, 62). Thus the function of VEGF within adult tissues and organs is likely to involve multiple cell types and be controlled at several levels. Consequently, it is not surprising that numerous studies have implicated VEGF and VEGF family members as active participants in lung pathology, with both loss of VEGF function and excessive activity leading to dysfunction.

In the present study, we investigated the potential link between NE, a well-known mediator of lung injury, and VEGF. We observed that VEGF is subject to partial cleavage by NE, leading to the generation of a VEGFf with altered activity. Whereas intact VEGFf binds VEGFR1 and -2 and heparin, the NE-generated VEGFf shows a selective loss of binding to VEGFR2 and heparin while retaining VEGFR1
binding. Interestingly, the altered receptor binding translated to a loss of signaling potential in endothelial cells, as noted by reduced ERK1/2 activation, yet no loss of activity in RAW 264.7 monocyte/macrophage cells. Moreover, the NE-generated VEGFf showed enhanced ability to stimulate Akt phosphorylation. Although we do not yet know if the NE-generated VEGFf plays important roles in mediating the pulmonary response to injury, our findings suggest that the role of extracellular proteolytic processing of VEGF should be considered as this process is more fully studied.

The generation of bioactive VEGFf by NE was not reflective of digestion by all extracellular proteases, since MMP9 did not lead to VEGFf production. In addition, NE digestion did not appear to lead to the production of partially cleaved fragments with all growth factors/cytokines tested. Indeed, FGF2, which has been shown to be released from ECM storage sites by elastase injury (12), appeared to be significantly resistant to NE cleavage, whereas TNF-α and PDGF-AA showed somewhat distinct digestion profiles. Interestingly, NE digestion of PDGF-AA, a structural analog of VEGF, appeared to lead to the generation of a fragment similar to that observed with VEGF. It will be important to consider the potential functions of these processed forms of growth factors in mediating the pulmonary response to proteolytic injury.

VEGF165 is secreted by a variety of cell types in the lung, but a significant fraction remains bound to the ECM (59). ECM-bound VEGF has been shown to be released by plasmin cleavage, which generates a bioactive VEGFf (33). Recent findings have also shown that VEGF can be modulated by other proteases as well (2, 45). Here we show that VEGF bioavailability and activity might also be controlled by processing through the action of NE. Hence, at sites of tissue injury or inflammation, NE released by activated neutrophils will cause ECM degradation, and potentially the processing of VEGF to a form that will selectively activate cells via VEGFR1 (Fig. 10). Our finding that VEGF is processed to a
new form by NE is interesting in light of a number of recent findings that have identified cryptic protein activities generated by proteolytic attack on the ECM (16). In particular, a large number of endogenous angiogenesis inhibitors have been demonstrated to be derived by the proteolytic processing of other ECM proteins such as type XVIII collagen, type IV collagen, perlecan, and fibulins 1 and 5 (16, 38, 50, 54, 56, 81). Moreover, even nonprotein components of the ECM, such as hyaluronan and heparan sulfate, have been demonstrated to function alternatively as inhibitors or stimulators of angiogenesis depending on the size of the polysaccharide chain, indicating a mechanism of control related to ECM degradation (60, 69). Thus the proteolytic processing of ECM-resident VEGF might also have important implications for angiogenic control in response to tissue injury and damage.

Analysis of NE-produced VEGF by MS indicates that NE cleaves VEGF at its NH\textsubscript{2} and COOH terminus, with some suggestion that there might also be cleavage sites within the internal region of the protein chain as well (Fig. 2). Internal cleavage would predict the generation of fragments of lower molecular mass than the major fragment we observe under reducing SDS-PAGE; however, it is important to note that the highly disulfide-linked cystine knot structure might allow intrachain disulfide bonds to resist standard reduction conditions (51, 52). In any case, the cleavage within the canonical heparin-binding domain in the COOH terminus of VEGF (21) is consistent with the loss of heparin affinity of the VEGF compared with the intact VEGF\textsubscript{165}. The decreased affinity for heparin binding would potentially make NE-generated VEGF more diffusible such that the ECM-released fragments could stimulate cells (e.g., macrophages) at sites distant to the primary site of NE action. Thus the powerful proteolytic activity of NE might not only participate in enhancing migration of neutrophils by removing connective tissue, but it might also generate active VEGF which could contribute to further inflammatory cell recruitment and activation.

VEGF is lost the ability to bind VEGFR2, the main mediator of VEGF activities, whereas it has retained the ability to bind VEGFR1 (Fig. 7). The function of VEGFR1 has been an active topic of debate, with studies indicating that VEGFR1 functions mainly as a “decoy” receptor, preventing VEGF from binding to VEGFR2 on the vascular endothelium (58). However, the expression of VEGFR1 on nonendothelial cell types such as pulmonary epithelial cells and monocyte/macrophages suggests additional roles for this receptor. VEGF is a strong activator of ERK1/2 via VEGFR2 (35) in endothelial cells, and, consistent with this finding, VEGF did not cause ERK1/2 activation in the pulmonary endothelial cells when added alone.

Interestingly, when added together with intact VEGF\textsubscript{165}, VEGF appeared to potentiate ERK1/2 activation (Fig. 8A). This finding suggests that VEGF might shield intact VEGF from binding to VEGFR1, making it more available to mediate its effects through VEGFR2 on endothelial cells. A similar function has been suggested for the VEGFR1 ligand PIGF (47). However, when we investigated Akt activation, a major factor implicated in VEGF-mediated cell survival (28, 74), we were surprised to find that VEGF was a more potent activator of Akt compared with the intact VEGF (Fig. 8). These findings suggest that VEGF can signal via VEGFR1 to activate Akt in endothelial cells. Although VEGFR1 certainly shows impaired kinase activity compared with VEGFR2, site-directed mutagenesis has identified several phosphorylated residues in VEGFR1 capable of interacting with SH\textsubscript{2}-domain proteins such as phosphatidylinositol 3-kinase (34).

The observation that VEGF and PI GF are potent activators of monocyte/macrophage migration (1, 5) led us to investigate the effects of the NE-generated VEGF in the mouse macrophage/monocyte cell line RAW 264.7. RAW 264.7 cells express VEGFR1 with no detectable VEGFR2, and treatment of these cells with VEGF led to Akt and ERK1/2 activation, increased VEGF expression, and stimulation of chemotaxis. The ability of VEGF and intact VEGF\textsubscript{165} to activate ERK1/2 in RAW 264.7 cells, in a VEGFR2-independent manner, suggests that distinct signaling pathways are present in these cells that are not in endothelial cells. Thus it is possible that VEGF can activate a range of activities in nonendothelial cells within elastase-damaged lungs via cell type-specific VEGFR1 signaling.

In the present study, we report that VEGF\textsubscript{165} is a substrate of NE and that the partial digestion of VEGF produces a fragment with altered activity and potentially altered function compared with intact VEGF. Although it is not clear what role NE-generated VEGF play in mediating the tissue response to injury in the lung, it is intriguing to speculate that the functional response to VEGF might relate to the ratio of VEGF to intact VEGF. Whereas NE-digestion of ECM might release both fragmented and intact VEGF through the action of NE directly on VEGF and indirectly on VEGF-binding sites [e.g., HSPG (10, 12)]; it is possible that, under certain conditions, VEGF can function to enhance the activity of VEGF on endothelial cells, and further function by diffusing to distant sites to recruit macrophages to the site of injury (Fig. 10).

However, chronic or excessive elastolysis might lead to com-
complete conversion of VEGF to VEGFF, with the concomitant loss of critical endothelial activities and excessive inflammatory cell activation. A more complete understanding of the role of VEGF within the lung will need to consider the possible function of proteolytic fragments of VEGF.

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