ORIGINAL RESEARCH

PR1P Stabilizes VEGF and Upregulates Its Signaling to Reduce Elastase-induced Murine Emphysema

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Abstract

Emphysema is a progressive and fatal lung disease with no cure that is characterized by thinning, enlargement, and destruction of alveoli, leading to impaired gas exchange. Disease progression is due in part to dysregulation of VEGF (vascular endothelial growth factor) signaling in the lungs and increased lung-cell apoptosis. Here we asked whether PR1P (Prominin-1–derived peptide), a novel short peptide we designed that increases VEGF binding to endothelial cells, could be used to improve outcome in *in vitro* and *in vivo* models of emphysema. We used computer simulation and *in vitro* and *in vivo* studies to show that PR1P upregulated endogenous VEGF receptor-2 signaling by binding VEGF and preventing its proteolytic

degradation. In so doing, PR1P mitigated toxin-induced lung-cell apoptosis, including from cigarette-smoke extract *in vitro* and from LPS *in vivo* in mice. Remarkably, inhaled PR1P led to significantly increased VEGF concentrations in murine lungs within 30 minutes that remained greater than twofold above that of control animals 24 hours later. Finally, inhaled PR1P reduced acute lung injury in 4- and 21-day elastase-induced murine emphysema models. Taken together, these results highlight the potential of PR1P as a novel therapeutic agent for the treatment of emphysema or other lung diseases characterized by VEGF signaling dysregulation.

Keywords: vascular endothelial growth factor; apoptosis; Prominin-1–derived peptide; emphysema; LPS

Emphysema is a chronic, debilitating, and fatal lung disease that lacks a therapeutic to prevent its progression or to restore lung tissue to health (1). It is characterized by gradual thinning, enlargement, and destruction of alveoli, leading to impaired oxygenation and carbon-dioxide retention (2). Although the mechanisms are incompletely understood, evidence suggests that disease progression is due in part to 1) chronic lung inflammation leading to increased secretion of proteases by alveolar neutrophils and macrophages within the lung parenchyma (3), 2) dysregulation within the lungs of vascular endothelial

growth factor (VEGF) and VEGFR2 (VEGF receptor-2) (4), and 3) increased lung endothelial and epithelial cell apoptosis (5). Recent interest in emphysema has focused on developing pharmaceuticals that target VEGF, as it is known to play a significant role in the lung in maintaining tissue homeostasis, is curiously expressed at 500-fold-higher concentrations in normal lungs compared with serum (6), and is directly or indirectly implicated in mediating the aforementioned disease mechanisms.

VEGF₁₆₅ (a 165–amino acid isoform of VEGF-A, henceforth referred to as VEGF)

is a ubiquitous angiogenic and survival factor that binds VEGFR1 and VEGFR2, as well as NRP-1 (neuropilin-1) (7). VEGFR1 and VEGFR2 are expressed to varying degrees on lung endothelial and epithelial cells and on alveolar macrophages (8). *In vitro* emphysema models using cigarette-smoke extract (CSE) or LPS as cell toxins show that VEGF modulates lung-cell apoptosis and cell survival in part through signaling through VEGF receptors and downstream activation of PI3K/Akt (9). Whether VEGF dysregulation leads to emphysema or is a byproduct of disease process is unknown.

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We recently showed that Prominin-1, a pentaspan transmembrane glycoprotein originally identified as a unique stem-cell surface marker but later found to be expressed in many mature adult human cells (10, 11), bound VEGF and potentiated its proangiogenic and antiapoptotic activity (12). We have subsequently designed a novel 12-amino acid peptide, PR1P (Promin-1-derived peptide), whose sequence is derived from an extracellular VEGF-binding domain of Prominin-1 that also binds VEGF, augments VEGF binding to the VEGFR2 and NRP-1 receptor in endothelial cells *in vitro* (12), and upregulates VEGF-dependent angiogenesis in multiple *in vitro* and *in vivo* models (13). The mechanism of action of PR1P remains unclear. Because VEGF regulation of apoptosis is primarily via downstream VEGFR2 signaling pathways (14), we hypothesized that PR1P would mitigate toxin-mediated apoptosis through upregulation of VEGFR2 signaling and in so doing could be used as a therapeutic in emphysema.

Here, we determined that PR1P activates VEGFR2-dependent signaling and explored its mechanism of action by employing molecular binding studies and three-dimensional (3D) computer simulation. Simulation studies revealed important interactions between PR1P and VEGF that predicted that PR1P binding of VEGF may stabilize VEGF from proteolytic degradation, which we demonstrated *in vitro* and *in vivo*. This was followed with studies showing that PR1P could be used to mitigate lung-cell apoptosis induced by CSE *in vitro* and LPS *in vivo* in mice. In addition, inhaled PR1P led to significantly increased VEGF concentrations in murine lungs within 30 minutes that remained greater than twofold above that of control animals 24 hours later. Finally, inhaled PR1P reduced elastase-induced emphysema in 4- and 21-day murine emphysema models. Together, these findings suggest that PR1P could be used as a novel therapeutic in emphysema by stabilizing endogenous VEGF signaling.

Methods

Inhalation Studies for pVEGFR2 Analysis by FACS

Eight-week-old female C3H/HENCrI (Charles River Laboratories) mice (22 ± 2 g)

were placed in a whole-animal nebulization chamber ($14 \times 5 \times 8$ cm) and allowed to spontaneously inhale nebulized PR1P, SP (scrambled peptide; 300 μ g/ml), or VEGF (500 ng/ml) in 3 ml of 0.9% normal saline for 20 minutes (Proneb Ultra II Nebulizer, Pari Respiratory Equipment). Animals were killed 30 minutes after nebulization, lungs were harvested, and lung tissue was minced using surgical scissors and suspended in 3 ml of serum-free bronchial epithelial-cell growth basal medium containing Liberase (2 mg/ml; Sigma-Aldrich), Dispase (5 U/ml; Stemcell Technology), and DNase (50 U/ml; Sigma-Aldrich) for 30 minutes at 37°C. Digested tissue was filtered (70- μ m mesh [Thermo Fisher Scientific]), and filtered cells were washed twice with FACS media (PBS containing 3% FCS) and centrifuged at 1,000 rotations/min for 5 minutes. Pelleted material was incubated for 90 seconds in erythrocyte-lysis buffer (Sigma-Aldrich), resuspended in FACS media, and prepared for FACS analysis (see data supplement).

Intratracheal Elastase Administration and Preparation of Lung Sections

Mice were anesthetized (ketamine [100 mg/kg] and xylazine [15 mg/kg] i.p.) and orally intubated with a 22-gauge plastic angiocatheter (Becton Dickinson) through which a dose of porcine pancreatic elastase (0.1 enzymatic activity U dissolved in 50 μ l of sterile normal saline; Fitzgerald), or NS (control) was delivered. Upon experiment conclusion, lungs were harvested and fixed with 10% formalin at 35 cm H₂O. The trachea was then ligated with a silk suture, and the specimen was preserved in 10% formalin. Five-micrometer lung sections were stained with hematoxylin and eosin. Qualitative assessment of the severity of morphological changes in whole-lung sections were assessed by a blinded histologist using an emphysema score of 0–5 (15). To quantify elastase-induced emphysema phenotype, we measured the mean linear intercept of lung sections using a system described by Knudsen and colleagues (16). Briefly, we used Image J software (Image J version 1.52q; National Institutes of Health) to overlay a grid containing 8 vertical and 10 horizontal lines onto each lung section image. Distances along the *x*-axis (in pixels) from each intersection of vertical and horizontal lines to the closest lung tissue were measured. If lung tissue was present underneath an

intersection, the distance along the *x*-axis from the far-right edge of the lung tissue under the intersection to the next nearest section of lung tissue was instead measured and used as a surrogate of the linear intercept for that point. We used a random pattern to collect 30–50 images from each hematoxylin and eosin-stained lung section. Images were assigned random numbers using the RAND function in Microsoft Excel (version 15.33; Microsoft Corporation), and an equal number of randomly selected images with randomly assigned numbers from each group were blindly analyzed for each experiment.

Results

PR1P Upregulates Endogenous VEGF Signaling through VEGFR2

We hypothesized that PR1P activity is mediated via upregulation of signaling through VEGFR2. To test this hypothesis, FACS was used to measure concentrations of phosphorylated VEGFR2 in the human-lung respiratory epithelial-cell line BEAS-2B in response to PR1P. As shown in Figures 1A and 1B, incubation of cells with PR1P significantly increased VEGFR2 phosphorylation compared with incubation with SP. To determine whether PR1P induction of VEGF signaling required VEGFR2 activation, cells were pretreated with the specific VEGFR2 inhibitor SU5416 or with vehicle alone as a control for 24 hours, and then pretreated cells were exposed to PR1P or to vehicle for 20 minutes. Cells were then harvested, and concentrations of phosphorylated VEGFR2 and AKT were assessed using FACS. Figures 1C–1F show that VEGFR2 inhibition significantly reduced the effect of PR1P on VEGFR2 and AKT phosphorylation, suggesting that PR1P activity required VEGF binding to and activation of VEGFR2. To determine whether PR1P inhalation could be used to activate endogenous VEGF signaling in animal lungs, mice were treated with nebulized SP, VEGF, or PR1P, and FACS analysis was performed on lung cells harvested from the animals 30 minutes after inhalation. As shown in Figures 1G and 1H, inhaled PR1P increased lung-cell VEGFR-2 phosphorylation compared with inhaled SP or VEGF. Importantly, we confirmed by Western blot that the PR1P-induced increase in lung-cell VEGFR2 phosphorylation occurred without changes in total VEGFR2

Figure 1. PR1P (Prominin-1–derived peptide) increases VEGF (vascular endothelial growth factor) signaling in lung cells *in vitro* and *in vivo*. (A) Representative FACS analysis of human bronchial epithelial (BEAS-2B) cells following incubation with VEGF, PR1P, or SP (scrambled peptide) showing increased phosphorylation of VEGFR2 (VEGF receptor 2) by PR1P. (B) Quantification of VEGFR2 phosphorylation from FACS experiments described in A. Data are mean + SEM ($n = 3$). $*P < 0.02$. (C and D) Representative FACS analysis of BEAS-2B cells following incubation with PR1P in the absence or presence of the VEGFR2 inhibitor SU5416 showing that increased phosphorylation of VEGFR2 (C) and AKT (D) requires signaling through VEGFR2. (E and F) Bar graphs showing percentages of cells in experiments described in C and D expressing pVEGFR2 (phosphorylated VEGFR2) (E) or pAKT (phosphorylated AKT) (F). $*P < 0.05$. (G) Representative FACS analysis of cells harvested from mouse lungs following inhalation of nebulized SP, VEGF, or PR1P showing increased pVEGFR2 after VEGF or PR1P inhalation. (H) Mean fluorescence intensity (MFI) of pVEGFR2 as a ratio of control (SP) from experiments described in G. $*P < 0.03$. Inhib = inhibitor. FSC = forward scatter.

concentrations (see Figure E1 in the data supplement). Together, these data suggested that PR1P activated endogenous VEGF signaling *in vitro* and *in vivo* but offered little insight into the PR1P mechanism. We next hypothesized that understanding how PR1P interacts with VEGF would provide insight into its mechanism of action.

To test this hypothesis, molecular biology and computer simulation were used to investigate the molecular interactions between PR1P and VEGF. Because there are no antibodies that recognize PR1P that could be used to identify binding interactions between PR1P and VEGF directly, and because PR1P is derived from a VEGF-binding region on Prominin-1, the binding interactions between Prominin-1 and VEGF were examined using molecular-biology studies to gain insight into where PR1P might bind VEGF. For these studies, peptides that were 12 amino acids long and contained overlapping sequences from the entire amino acid sequence of VEGF were tested for their affinity for binding to Prominin-1 using a peptide binding array. Here, nitrocellulose membranes with transferred short peptides containing the VEGF sequences were incubated with Prominin-1 and blotted with anti-Prominin-1 antibody (Figure 2A). Blots revealed that the four VEGF-derived peptides that displayed the greatest binding of Prominin-1 (Figure 2A) were each derived from the heparin-binding domain (HBD) of VEGF (Figure 2B). These findings suggested that Prominin-1 and therefore PR1P bind VEGF on or near its HBD. We next used 3D computational simulation and structural molecular modeling to provide molecular insight into binding interactions between Prominin-1 or PR1P and VEGF. The structural molecular modeling explored the interactions between Prominin-1_{K192-Q398} (a Prominin-1 fragment), which consisted of the second extracellular VEGF binding domain of Prominin-1 and that included the PR1P sequence (13), and VEGF_{A110-R165} or VEGF_{HBD} (the HBD-containing fragment of VEGF; Figures 3A and 3B). To begin, 3D structures of Prominin-1_{K192-Q398} (using PEP-FOLD3) were generated, and the best model was docked into the known crystal structure of the VEGF_{HBD} fragment (using ClusPro 2.0) (Figure 3A and Table E1). Figure 3A shows a close-up view of this interaction and illustrates that Q372 (glutamine at position 372) in

Figure 2. VEGF binds Prominin-1 via the heparin-binding domain (HBD). (A) Four 12-mer peptides (VP1–VP4 [VEGF-derived peptides 1–4]) displaying the greatest binding of Prominin-1 (among 179 peptides tested) were each derived from the VEGF HBD of VEGF, as shown in B. (B) A schematic representation of two 165-amino acid VEGF monomers linked by two s–s forming a VEGF₁₆₅ heterodimer. The blue regions represent the N amino acids 1–110, and the red regions represent the HBD C amino acids 111–165. The plasmin-cleavage sites are indicated by green arrows (between amino acids R109 [arginine at position 109] and A110 [alanine at position 110]). The amino acid sequences 106–109 (blue font) and the entire HBD (110–165, red font) are shown together with the location and sequences of the four overlapping 12-mer VEGF peptides VP1–VP4 (gray boxes described in A). Note the asterisk (*) in B indicates that R138 is identified as a crucial amino acid in the VEGF sequence responsible for binding to both prominin-1 and to PR1P (see text and Figure 3). C = carboxy-terminal; N = amino-terminal; s–s = disulfide bonds.

Prominin-1_{K192-Q398} (corresponding to residue Q4 in PR1P [i.e., DRVQRQTITVVA]) formed hydrogen bonds with residue R138 (arginine at position 138) of VEGF_{HBD}. Note that the PR1P sequence corresponds to D369–A380 (aspartic acid at position 369–alanine at position 380) in Prominin1_{K192-Q398} (purple in Figure 3A). Next, 3D structures of PR1P were generated, and the best model was docked into the 3D crystal structure of VEGF_{HBD} (Figure 3B). Interestingly, D1 in PR1P (DRVQRQTITVVA), which corresponds to residue D369 in Prominin-1_{K192-Q398}, also formed hydrogen bonds with R138 of VEGF_{HBD}. Together, these results suggested an important role for R138 in the VEGF_{HBD} that enabled its association with both Prominin-1_{K192-Q398} and PR1P. Importantly, VEGF degradation by proteases requires the

HBD, and the plasmin-cleavage site within the VEGF HBD contains the R138 (i.e., R136–A137–R138 in VEGF_{HBD} [17, 18]; see plasmin-cleavage site [green arrow] in Figure 2B). We therefore hypothesized that PR1P binding to VEGF at R138 might interfere with VEGF degradation by proteolytic enzymes such as plasmin and elastase. Note the concentrations of these two enzymes are elevated during acute and chronic inflammation seen in emphysema and other inflammatory lung diseases (19). To test this hypothesis, docking assays between VEGF_{HBD} and the 3D crystal structure of either elastase (Figure 3C) or plasmin were performed (Figure 3D). Interestingly, this modeling indicated that R138 of VEGF formed hydrogen bonds with Q187 located within a highly flexible loop in elastase (Figure 3C), and with G728 (glycine

at position 728) in plasmin (Figure 3D). Together, these results suggested that residue D1 of PR1P binding to VEGF (at R138) may interfere with both elastase and/or plasmin docking on VEGF. To test this, further simulation docking experiments between elastase or plasmin and the VEGF_{HBD} in the presence of PR1P were performed. As shown in Figures 3E and 3F, this modeling revealed that PR1P binding to VEGF_{HBD} indeed interfered with both elastase and plasmin binding to VEGF_{HBD} at R138, suggesting that PR1P may block elastase and plasmin from associating with VEGF at its proteolytic region. To support these simulation findings that revealed the critical importance of the first amino acid D1 on PR1P, a modified PR1P peptide that contained A1 instead of D1 was generated. As shown in Figures 3G–3I, this substitution (A1 for D1) almost completely reduced the ability of the modified peptide to augment VEGF binding to endothelial cells (Figure 3G) and to stimulate VEGF signaling in respiratory epithelial cells *in vitro* (Figures 3H and 3I) compared with PR1P. To test whether PR1P binding to VEGF protected VEGF from protease degradation, we incubated VEGF *in vitro* with elastase or with plasmin in digestion buffer at 37°C for 2 hours in the presence and absence of PR1P or SP and evaluated the sizes of resultant VEGF degradation products under nonreducing conditions by Western blot analysis. Note that elastase cleaves VEGF at both amino- and carboxy-terminal ends (17), and that plasmin cleaves VEGF at amino acid 110, both rendering VEGF fragments with approximate molecular mass of 35 kD (18). Interestingly, we found that PR1P significantly reduced the proportion of both elastase-generated (Figure 3J) and plasmin-generated (Figure E2) VEGF fragments. To determine whether PR1P protects VEGF from degradation by endogenous proteases *in vivo*, we harvested murine lungs at multiple time points between 30 minutes and 24 hours after exposing the animals to inhaled PR1P (or vehicle as control). As shown in Figures 3K–3M, Western blotting of whole-lung lysates revealed that inhalation of PR1P led to a significant increase in VEGF Dimer/Monomer ratios (Figures 3K and 3L) as well as VEGF dimer concentrations (Figure 3M) as early as 30 minutes after inhalation. Note that VEGF dimers represent the active form of VEGF and that covalent dimerization of VEGF is essential for effective receptor binding and biological activity (20).

Figure 3. Three-dimensional modeling suggests that PR1P blocks elastase and plasmin binding to the proteolytic region on VEGF. (A and B) Ribbon representations of Prominin-1_{K192-Q398} (a Prominin-1 fragment; blue), HBD-containing fragment of VEGF (VEGF_{HBD}; green), and PR1P (red). (A) Prominin-1_{K192-Q398} in a complex with VEGF_{HBD} (bottom left) with a close-up view of interactions within the orange square shown at top right. The region in Prominin-1_{K192-Q398} that contains the PR1P sequence is colored in purple, and residues forming H-bonds are colored in pink. Interacting residues between Prominin-1_{K192-Q398} and VEGF_{HBD} are shown and are color-coded according to the peptide to which they belong (see Table E1 for details). (B) VEGF_{HBD} in complex with PR1P. Residues R138 of VEGF_{HBD} and D1 (aspartic acid at position 1) of PR1P form H-bonds (pink; see Table E2). (C–F) Ribbon representations of VEGF_{HBD} (green) in complex with elastase or plasmin (blue) in absence or presence of PR1P (red). (C and D) VEGF_{HBD} in complex with elastase (C) or plasmin (D). Note that residue R138 of VEGF_{HBD} forms H-bonds with (C) Q187 (glutamine at position 187) of elastase and with (D) G728 (glycine at position 728) of plasmin. (E and F) VEGF_{HBD} in complex with elastase or plasmin in the presence of PR1P showing that PR1P binding to VEGF_{HBD} prevents elastase (E) and plasmin binding (F) to the proteolytic cleavage region of VEGF_{HBD} (i.e., keeps Q187 on elastase and G728 on plasmin from binding to R138 on VEGF_{HBD}). Note that G728 is not visible in F in this view because of protein folding. (G) Bar graph showing cell radioactivity amounts after incubation with ¹²⁵I-VEGF in the absence (control) or presence of PR1P (DRVQRQTITVVA) or modPR1P (modified PR1P; ARVQRQTITVVA), in which D1 is substituted with A1 (red). Note the significant reduction in ¹²⁵I-VEGF binding after peptide modification. Data are mean + SEM (n = 3). *P < 0.05. (H) Representative FACS analysis of BEAS-2B cells after incubation in the absence or presence of PR1P or modPR1P. Note modPR1P does not augment AKT and VEGFR-2 phosphorylation. (I) Quantification of VEGFR2 and AKT phosphorylation from FACS experiments described in H. Data are mean + SEM (n = 3). *P < 0.003. (J) Representative Western blot analysis of VEGF protein (VEGF dimer) incubated (2 h) in the conditions indicated, showing that PR1P reduces the proportion of elastase-induced VEGF degradation products. n = 5. (K–M) PR1P protects VEGF from degradation *in vivo*. Representative Western blot of whole-lung lysates from individual mice 30 minutes after inhalation of PR1P or SP showing that PR1P increases VEGF dimer:monomer ratios as well as VEGF concentrations (n = 3). Readers may view the original uncut gels for K in the data supplement (Figure E3). (L) Bar graph showing quantification of the VEGF dimer:monomer ratios from the blot shown in K. *P < 0.003. (M) Bar graph showing quantification of the density of the VEGF dimers relative to the standards in the blot shown in K. *P < 0.0003. Blots were analyzed using Image J software.

Figure 3. (Continued).

ELISA-based VEGF assays of lysates from whole-lung digests at 4 and 24 hours after PR1P inhalation showed that VEGF concentrations in PR1P-treated lungs were significantly elevated at both 4 and 24 hours compared with control-treated lungs at 0 h (Figure E3). These findings suggest that PR1P binding to endogenous VEGF increases its concentrations by disturbing the balance of VEGF production and degradation. Together, these simulation and molecular-biology studies strongly support the hypothesis that PR1P augments VEGF signaling in part by binding to and stabilizing endogenous VEGF from degradation.

PR1P Decreases Toxin-induced Lung-Cell Apoptosis *In Vitro* and *In Vivo*

We recently showed that knockdown of Prominin-1 resulted in increased apoptosis of melanoma cells *in vitro* (12). Because PR1P is derived from Prominin-1 and was shown to upregulate endogenous VEGFR2 signaling, which mediates apoptosis (7), we hypothesized that PR1P could be used to decrease toxin-induced apoptosis of lung

cells *in vitro* and *in vivo*. To test this hypothesis, the effect of PR1P on CSE-induced apoptosis in lung respiratory epithelial cells (BEAS-2B) *in vitro* (21) was first characterized. To begin these studies, cells were incubated with increasing concentrations of CSE for 48 hours, and 3% CSE was selected as an optimal concentration for all studies, as it arrested cell proliferation and had a minimal effect on cell death (Figure E4). TUNEL staining of cells exposed to CSE for 24 hours in the presence or absence of PR1P revealed that PR1P reduced the proportion of CSE-induced apoptotic cells (data not shown). This effect was quantified through FACS analysis of similarly treated cells, which revealed that CSE-induced cell apoptosis was significantly mitigated by PR1P (Figures 4A and 4B). Because BEAS-2B cells are immortalized human bronchial epithelial cells and much of the damage seen in emphysema is to the distal alveolar epithelium, the effect of PR1P was evaluated for mitigating smoke-induced apoptosis in human alveolar epithelial cells. Figure E5 shows that PR1P also similarly reduced apoptosis in alveolar epithelial cells

exposed to CSE. Considering that we showed earlier that PR1P was bioactive when inhaled into living mice (Figures 1G and 1H), we next sought to determine whether PR1P protected lung cells from LPS-induced apoptosis *in vivo* (22). As shown in Figure 4, inhalation by live mice of PR1P after LPS inhalation significantly reduced LPS-induced lung-cell apoptosis (Figures 4C–4F), specifically of lung epithelial cells (Figures 4G and 4H). To determine whether PR1P similarly reduced LPS-induced lung-cell necrosis, a subset of lung cells was costained in LPS and FACS experiments parallel to those described above with caspase 3 and with propidium iodide to identify cell necrosis. Figure E6 shows that PR1P reduced the percentage of both LPS-induced lung-cell apoptosis and LPS-induced necrosis. Taken together, these results suggest that PR1P protects lung cells *in vitro* and *in vivo* from toxin-induced lung-cell apoptosis and necrosis.

PR1P Mitigates Elastase-induced Emphysema

To characterize the ability of inhaled PR1P to delay the onset of, or reverse the

Figure 4. PRIP reduces toxin-induced lung-cell apoptosis *in vitro* and *in vivo*. (A) Representative FACS analysis of BEAS-2B cells treated with cigarette-smoke extract (CSE) for 24 hours in the presence of SP or PR1P and stained with TUNEL showing that PR1P reduces CSE-induced apoptosis. (B) Quantification of apoptosis (TUNEL MFI) from FACS experiments described in A. Data

pathology in, emphysema, an established murine emphysema model was used in which the emphysema phenotype is induced by intratracheal injection of porcine pancreatic elastase (Elastase). To establish a timeline of lung injury in our model, C3H/HENCrI adult mice were treated with intratracheal Elastase and assessed for the degree of emphysema phenotype (lung injury) on lung histology at 24, 48, 72, and 96 hours after treatment. In agreement with reports in the literature, we found that Elastase induced characteristic changes in lung architecture, including destruction of the alveolar walls and alveoli enlargement, as early as 24 hours after injection (Figure 5A and Reference 23) that were indistinguishable from injuries seen at 4 days. To determine whether PR1P therapy could be used as a therapeutic in these animals over a period of 4 days, daily inhaled treatments of PR1P or control vehicle were initiated beginning at 24 hours after Elastase administration. *Qualitative* scoring of histological lung sections at 4 days by a histologist blinded to treatment indicated that PR1P significantly mitigated damage to lung architecture (Figure 5B). Blinded quantification of airspace enlargement determined by mean-linear-intercept analysis (16) similarly indicated that PR1P significantly reduced elastase-induced alveolar damage (Figure 5C). Interestingly, the distributions of chord lengths were skewed in all treatment groups as expected, but the curves of the control- and PR1P-treated groups had slimmer and higher peaks compared with Elastase groups treated with vehicle alone (Figure 5D). In select animals, pulmonary function testing was performed on Day 4 before necropsy using the flexiVent (SQIREQ) system and found a significant reduction in elastase-induced increased compliance by PR1P inhalation (Figure 5E). To determine whether PR1P treatment can be administered repeatedly and over a longer term as an emphysema therapeutic, 21-day elastase-injury experiments were conducted. Mice treated with intratracheal elastase as described above were then also treated with inhaled PR1P or control vehicle every other day for 21 days. Similar to analyses of the 4-day elastase studies, qualitative analysis of lung sections by a blinded histologist revealed a significant reduction by inhaled PR1P of elastase-induced damage to lung architecture at 21 days (Figure E7). In these animals,

A **B** **C** **D** **E** **F**

Figure 5. Inhaled PR1P improves lung morphology in elastase-induced emphysema in mice. (A) Representative images of hematoxylin and eosin (H&E)-stained lung sections obtained from mice 4 days after intratracheal injections of normal saline or elastase, followed by daily treatment of inhaled SP or PR1P. Scale bar, 100 μ m. (B and C) Bar graphs showing results of blinded qualitative emphysema score (B) or quantitative linear-intercept-length analyses of whole-lung sections (C) from experiments described in A, indicating significant reduction in emphysema phenotype by inhaled PR1P. $n = 4$ experiments; 3 mice per group; $*P < 0.05$. Note that the measured mean linear intercepts of the control, elastase + vehicle, and elastase + PR1P images at 4 days shown in A are 42.1, 101.6, and 80.7, respectively. (D) Representative distribution analysis of line-segment lengths obtained from wall-to-wall measurements of H&E-stained images of mouse lungs. Elastase treatment is associated with a wider and lower peak in intercept length distribution. Treatment with PR1P returns the distribution toward normal. (E and F) Bar graphs showing that inhaled PR1P reduces the elastase-induced increase in lung compliance at 4 days (E, n of 7 [control] or 13 [elastase and elastase/PR1P]; $*P < 0.03$) and at 21 days (F, n of 4 [control] or 10 [elastase and elastase/PR1P]; $*P < 0.03$) after injury.

pulmonary parameters were also analyzed with the flexiVent system on Day 21 before necropsy to measure lung compliance. Here, we similarly found that inhaled PR1P

significantly reduced the increase in compliance from elastase injury (Figure 5F). These data suggest that PR1P can be an effective therapeutic to reduce

early elastase-induced lung injury and that PR1P can be given repeatedly over a longer term to reduce lung injury seen during more chronic stages of disease. Together,

Figure 4. (Continued). are mean $\pm$ SEM ($n = 3$). $*P < 0.05$ and $**P < 0.01$. (C and D) Representative FACS analyses of cells harvested from the lungs of mice 24 hours after treatment with nebulized LPS followed by treatment with inhaled SP or PR1P showing that PR1P reduces cell apoptosis identified using caspase-3 (C) and annexin-V markers (D). (E and F) Quantification of apoptosis from FACS experiments described in C and D using caspase-3 (E) or annexin-V (F). Data are mean $\pm$ SEM ($n = 4$). $*P < 0.02$ in E and $*P < 0.05$ in F. (G) Representative FACS analysis of epithelial gated cells (using anti-cd326 as a marker), harvested from the lungs of mice treated as described in C, showing that PR1P reduces lung epithelial-cell apoptosis identified using caspase-3. (H) Quantification of apoptosis from FACS experiments described in E. Data are mean $\pm$ SEM ($n = 6$). $*P < 0.03$.

Figure 6. Schematic of experimental summary and proposed mechanism of PR1P-enhanced VEGF signaling. (A and B) PR1P is a 12-mer peptide (purple dots in B) whose amino acid sequence was derived from a VEGF-binding region (purple dots in B) on the second extracellular domain of Prominin-1 (blue in A), a 5-transmembrane spanning glycoprotein with angiogenic properties. VEGF (green; shown in the active dimeric form) contains an HBD (orange) at the C end of each monomer. VEGF binding to the VEGFR induces receptor dimerization and autophosphorylation, phosphorylation of AKT, and VEGF-mediated survival signaling through AKT. (B) PR1P binds VEGF within the HBD, which leads to increased VEGF binding to VEGFR and upregulation of VEGF signaling. (C) Proteases (such as elastase or plasmin; shown as scissors) cleave VEGF near or within the HBD into smaller degradation products with altered VEGFR binding properties (shown here for simplicity to not bind the VEGFR), leading to impaired VEGF signaling. (D) PR1P binding to VEGF within the HBD stabilizes VEGF by blocking its binding by proteases, resulting in reduced VEGF degradation, enhanced VEGF binding to VEGFR, and upregulation of VEGF signaling. pAKT = phosphorylated AKT; pVEGFR = phosphorylated VEGFR.

these findings highlight the potential for PR1P as a therapeutic for human emphysema.

Discussion

Here, we show that PR1P, a novel VEGF-binding peptide (13, 24), upregulated endogenous VEGF signaling through VEGFR2, reduced cigarette smoke- and LPS-induced lung-cell apoptosis *in vitro* and *in vivo*, respectively, and reduced acute lung injury in 4-day and 21-day elastase-induced murine emphysema models. Molecular-biology studies supported our computer simulation-generated hypothesis that PR1P binding to VEGF along the VEGF HBD prevents binding of VEGF by proteolytic enzymes to stabilize it from proteolytic degradation, enabling signaling upregulation. A schematic summarizing our findings and a hypothetical model of the effect of PR1P on VEGF signaling is presented in Figure 6.

The main objective of the various murine emphysema models is to best mimic

human disease. Importantly, different models each yield multiple characteristic features of the disease, but none reproduce all the features collectively. Many models, and in particular the murine cigarette-smoke model, better model early-stage emphysema (25). Elastase has been used extensively to induce severe emphysema in multiple animal models and is widely considered to be the best available model to examine severe chronic obstructive pulmonary disease in mice (26). We chose the Elastase model because we were interested in characterizing the effect of PR1P in both early and severe emphysema.

We found that PR1P binding to the VEGF HBD blocked VEGF degradation by plasmin and elastase *in vitro* and increased VEGF binding to and signaling through VEGFR2. Remarkably, we found that inhaled PR1P led to an increase in VEGF concentrations in the lungs in as little as 30 minutes and that VEGF concentrations remained elevated at 24 hours after inhalation. Also, VEGF Dimer/Monomer ratios in the lungs were significantly elevated at 30 minutes after inhalation.

Collectively, these data suggest that PR1P binding to VEGF likely leads to an imbalance between VEGF production and degradation factors, and by preventing VEGF degradation, PR1P promotes VEGF binding to receptors and VEGF signaling. VEGF signaling within tissue microenvironments *in vivo* is mediated by local factors that control gene transcription (27), mRNA stabilization (28), and translational (29) and post-translational protein degradation and modification. Disturbances in concentrations or activities of these factors during injury or disease induces severe VEGF dysregulation within tissues that can be long-lasting (30, 31).

The VEGF HBD plays a central role in mediating VEGF signaling within the tissue microenvironments, as it may determine VEGF isomer receptor-specific binding, localization, and signaling properties (32) and may serve as well as a binding site for proteases. Protease binding of the HBD cleaves VEGF into smaller VEGF isomers or degradation products with altered receptor binding and signaling properties (33). VEGF signaling in the lungs is

particularly vulnerable to proteases such as plasmin and elastase, which are secreted by inflammatory cells, and so VEGF signaling may become severely dysregulated during periods of acute or chronic inflammation, such as in emphysema. Our simulation experiments indicated that amino acid R138 on VEGF, which is necessary for plasmin binding within the VEGF HBD (i.e., the second R in the RAR plasmin-cleavage region; Figure 2), also formed high-energy bonds with Q187 of elastase within a highly flexible loop in elastase. These simulation data led us to hypothesize that PR1P binding to R138 on VEGF would block access of both plasmin and elastase to the VEGF HBD and would stabilize VEGF from degradation. Results of molecular-biology experiments were consistent with this hypothesis and indeed showed that PR1P binding to VEGF mitigated its digestion by proteases. Lauer and colleagues (34) showed that wound fluid from chronic human leg ulcers contained high concentrations of plasmin, with increased plasmin activity, increased VEGF degradation, and diminished VEGF concentrations and signaling. The plasmin in this fluid degraded wild-type VEGF *in vitro* but did not degrade a recombinant proteolysis-resistant VEGF mutant in which the plasmin binding site within the HBD was altered (34). The authors surmised that blocking protease binding to the HBD on VEGF to control its proteolysis in the chronic wound environment might be a key strategy to increase VEGF signaling and improve wound healing.

PR1P may impact inflammation and lung injury indirectly by binding VEGF and preventing its degradation by proteases. A recent report by Kurtagic and colleagues (32) showed that fVEGF (VEGF degradation fragments) generated by neutrophil elastase cleavage of VEGF induced macrophage and endothelial progenitor-cell migration *in vitro*. Neutrophil elastase released from neutrophils is believed to upregulate further neutrophil migration into the lungs in emphysema (35). We hypothesize that PR1P binding VEGF prevents the formation of fVEGF, and in so doing, PR1P indirectly mitigates inflammatory-cell recruitment into injured lungs and mediates lung inflammation. We are currently investigating this hypothesis in focused studies in our laboratory. Results from the literature support our hypothesis

that PR1P binding of VEGF on its HBD may stabilize VEGF within the inflamed lung to both preserve VEGF concentrations and redirect VEGF signaling toward VEGFR2. Furthermore, soluble Prominin-1 significantly reduced VEGF binding to VEGFR1 and NRP-1 *in vitro* (12), suggesting that binding VEGF by proteins near or on the HBD may significantly alter VEGF-receptor binding properties. Our more recent results showing that PR1P enhanced VEGF binding *in vitro* to VEGFR2 and to NRP-1 compared with VEGFR1 (14) provide evidence that this can be achieved using small binding peptides that may more efficiently alter or stabilize VEGF binding features, even in the absence of proteolytic degradation. Our studies lend credence to the importance of the HBD and its interaction with PR1P. As such, we would predict that the longer isoforms that contain the HBD (i.e., isoforms with 165 or more amino acids) would bind PR1P to varying degrees and that the tertiary structure of the longer isoforms might hinder interactions with the peptide. We predict PR1P to have no effect on VEGF₁₂₁. We recently found that minor modifications (single amino acid replacement or deletion) to the primary structure of PR1P led to loss of function (13). In separate unpublished experiments, we found that pegylation, methylation, circularization of the peptide, or tagging of the peptide with biotin or fluorescent labels all dramatically reduced the ability of the modified peptide to bind VEGF and/or enhance VEGF binding to endothelial cells. As a direct result of these properties, we have been unable to localize PR1P in the lung after inhalation or to determine its *in vivo* binding partners. Future work in our laboratory will use computer simulation to guide further studies to better characterize the effect of PR1P on VEGF isoform-specific signaling in the lungs.

We previously showed that knockdown of Prominin-1 expression in melanoma cells led to increased cell apoptosis (12). Here we found that PR1P binding to VEGF led to enhancement of VEGF binding to VEGFR2, increased VEGFR2 signaling, and similarly led to mitigation of toxin-induced apoptosis. We showed that inhaled PR1P reduced LPS-induced apoptosis of cells harvested from whole lungs, specifically in lung epithelial cells (Figure 4). This is important because apoptosis of lung epithelial cells in the alveolar wall is a

hallmark feature of emphysema (36). The lungs harbor many cell types, including epithelial, clara goblet, and neuroendocrine cells; different types of vascular endothelial, smooth muscle, and adventitial fibroblasts; and various types of immune cells that reside in the lungs or migrate into the lungs during inflammation. We focused on exploring the role of PR1P in lung epithelial-cell behavior for a number of reasons. First, epithelial cells are the main source of VEGF expression in the lungs (5, 37) and play a critical role in lung-cell survival (4). Third, epithelial cells cover ~90% of the alveolar surface area and serve as the initial barrier to the pathogens from the outside world (38). Interestingly, because lung epithelial cells comprise only 3–5% of the total lung-cell population (39), our data suggest that the effect of PR1P on lung-cell apoptosis must also include an effect on nonepithelial cells, including those recruited to the lungs after LPS stimulation, such as inflammatory cells (40). Lung endothelial, epithelial, and alveolar macrophages all express VEGF receptors to varying degrees (41), as do neutrophils and other inflammatory cells (8) and therefore could be targeted by PR1P. Understanding how PR1P affects each of these cell populations will provide valuable information necessary to develop a PR1P-based therapeutic for emphysema and other VEGF-dependent lung diseases.

We initiated inhaled PR1P therapy in both the 4-day and 21-day injury models beginning at 24 hours after intratracheal elastase administration. The half-life of exogenous elastase *in vivo* is short (~50 min), and single doses of elastase inhibitors have been shown to be effective in mitigating elastase-induced injury only when given immediately before or within 4–8 hours after elastase administration (42). Thus, elastase is required to initiate the process of connective-tissue destruction in this model, whereas disease progression is due to subsequent inflammation and lung remodeling in the absence of active exogenous elastase. We surmised from computer simulation that PR1P binding VEGF prevented elastase binding, and although PR1P was shown here to inhibit VEGF degradation, we did not find that PR1P inhibited elastase *per se* (Figure E8). These considerations are important because PR1P treatment at 24 hours after elastase treatment would not directly interfere with

the ability of elastase to initiate lung injury in these models. PR1P would, however, be effective in blocking any newly deposited elastase by inflammatory cells that are recruited to the lung after initiation of lung injury.

Finally, reduced VEGF and VEGFR-2 concentrations *in vivo* and *in vitro* are associated with pulmonary endothelial and epithelial cell apoptosis in emphysema models (43). These and similar findings have led many to believe that overexpression of VEGF in, or delivery of exogenous VEGF to, the lungs could be curative in emphysema (44). Enthusiasm for this type of therapy was tempered, however, by animal studies using systemic VEGF therapy (45) that were complicated by toxicity (46, 47). However, there are no reports of studies in humans or animals using lung-targeted or systemic VEGF therapy that induced toxicity in the lungs. In addition, to our knowledge, there are no studies reporting significant toxicity related to VEGF therapy in humans, and there are

several that report a lack of tumors or other toxicities many years after therapy (48–50). Although we found that inhaled PR1P reduced acute lung injury in our short- and longer-term murine elastase-induced emphysema models, our studies were not designed to evaluate systemic effects or toxicity from PR1P therapy. Nevertheless, we did not observe ill effects from PR1P inhalation in our animals (data not shown). Our results therefore raise the possibility that a strategy to use targeted PR1P therapy to upregulate endogenous VEGF signaling in the lungs *in vivo* may be effective in the lungs and may avoid systemic toxicity seen in animals receiving alternative and more conventional forms of VEGF therapy. It will be necessary to delineate the fate and pharmacokinetics of PR1P in evaluating its toxicity potential during drug development, and before testing in humans, should the peptide prove therapeutic in larger animal studies. Because of its small size, PR1P could also be more easily packaged as cargo inside nanometer- or micron-

sized storage vehicles for tissue- or cell-targeted therapy, which may further limit toxicity.

In summary, our findings suggest that PR1P could play a critical role in reducing apoptosis in emphysema or other lung or systemic diseases characterized by VEGF signaling dysregulation by stabilizing and upregulating endogenous VEGF. Our studies provide a proof of principle for a novel approach using small targeting peptides to enhance the activity of endogenous VEGF to enhance lung-cell and tissue survival from acute or chronic injury. These studies also support a role for short-sequence peptides in modulating growth-factor signaling in general. ■

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