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BRIEF COMMUNICATION

A novel strategy to enhance angiogenesis in vivo using the small VEGF-binding peptide PR1P

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Abstract Therapeutic angiogenesis is an experimental frontier in vascular biology that seeks to deliver angiogenic growth factors to ischemic or injured tissues to promote targeted formation of new blood vessels as an alternative approach to surgical revascularization procedures. Vascular endothelial growth factor (VEGF) is a potent angiogenic signal protein that is locally upregulated at sites of tissue injury. However, therapies aimed at increasing VEGF levels experimentally by injecting VEGF gene or protein failed to improve outcomes in human trials in part due to its short half-life and systemic toxicity. We recently designed a novel 12-amino acid peptide (PR1P) whose sequence was derived from an extracellular VEGF-binding domain of the pro-angiogenic glycoprotein prominin-1. In this study, we characterized the molecular binding properties of this novel potential therapeutic for targeted angiogenesis and provided the foundation for its use as an angiogenic molecule that can potentiate endogenous VEGF. We showed that PR1P bound VEGF directly and enhanced VEGF binding to endothelial cells and to VEGF receptors VEGFR2 and neuropilin-1. PR1P increased angiogenesis in the murine corneal micropocket assay when combined with VEGF, but had no activity without added VEGF. In addition, PR1P also enhanced angiogenesis in murine choroidal neovascularization and wound-healing models and augmented reperfusion in a murine hind-limb ischemia model. Together our data suggest that PR1P enhanced angiogenesis by potentiating the activity of endogenous VEGF. In so doing, this novel therapy takes advantage of endogenous VEGF gradients generated in injured tissues and may improve the efficacy of and avoid systemic toxicity seen with previous VEGF therapies.

Keywords VEGF · prominin-1 · Angiogenesis · Pro-angiogenesis therapy · Peptide · PR1P

Introduction

Angiogenesis, the development of new blood vessels, is vital to the repair of ischemic or injured tissue, and thus, much effort has been invested recently in designing effective pro-angiogenic therapies [1]. The expression of the potent angiogenic protein vascular endothelial growth factor (VEGF) transiently increases locally at sites of tissue injury and ischemia such as in myocardial infarction, limb ischemia in peripheral artery disease, and in ulcers. As a result, VEGF has been implicated as a key regulator in restoring injured tissue to health. Adequate blood flow is crucial for normal tissue function as well as for tissue recovery, and thus, therapeutic angiogenesis is now regarded as a promising experimental frontier in vascular biology. Historically, this new experimental therapeutic modality has involved the delivery of angiogenic factors such as VEGF directly to sites of tissue ischemia or injury, and certain success in animal experiments has raised hope

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that it could ultimately serve as an alternative approach to surgical revascularization procedures.

The human VEGF gene family includes VEGF-A, -B, -C, -D, -E, and placenta growth factor [2]. Alternative exon splicing produces multiple isoforms of VEGF proteins with varying biological and functional specificity [3, 4]. VEGF isomers bind two tyrosine kinase receptors, VEGF receptor 1 (VEGFR1) and VEGF receptor 2 (VEGFR2), as well as neuropilin-1 (NRP-1), in isoform-specific manner [5]. VEGF-A_{165} is the most studied isoform of the VEGF family (henceforth referred to as VEGF). VEGF has been shown to be important in tissue healing by promoting the early events in angiogenesis, particularly vascular endothelial cell migration [6], vascular endothelial and epithelial cell proliferation and survival, and blood vessel formation [7]. How VEGF mediates tissue revascularization and repair and how it may be pharmaceutically manipulated to prevent disease or accelerate recovery are the subject of intense investigation. Despite advancements in treatment strategies in animal studies, concerns for using this therapy in humans linger due to a risk of toxicity from VEGF, including edema, and aberrant angiogenesis and fibrosis when delivered systemically [8, 9]. In a murine hind-limb ischemia model, intramuscular injection of VEGF was associated with increased limb amputation rates [10]. Several recent clinical trials in patients with chronic ischemic pathologies including coronary and peripheral artery disease and ischemic ulcers [11–13] have failed to show a benefit of VEGF gene or protein therapy. The lack of efficacy in these circumstances has been attributed to the short half-life of VEGF in vivo, insufficient uptake of VEGF by organs after targeted or systemic treatment, desensitization of chronically ischemic tissues to growth factor treatment, and localized edema of affected organs [14].

We recently discovered that the pentaspan transmembrane glycoprotein prominin-1 binds VEGF and potentiates its anti-apoptotic and pro-angiogenic activities in vitro and in vivo [15]. Following this discovery, we set out to generate short peptides comprised of amino acid sequences contained within prominin-1 in an effort to generate VEGF-binding peptides with either anti- or pro- angiogenic properties. We found that one of the peptides that we generated, which we named prominin-1-binding peptide, or PR1P, significantly increased VEGF binding to endothelial cells. Here we tested the hypothesis that PR1P could be used to augment tissue angiogenesis and improve outcome from tissue injury by potentiating *endogenous* VEGF.

In these studies, we showed that PR1P, a short peptide incorporating 12 amino acids from an extracellular VEGF-binding domain of prominin-1, bound VEGF and increased VEGF binding to endothelial cells in vitro and specifically to VEGFR-2 and to NRP-1. Importantly, PR1P alone displayed no activity in the murine corneal micropocket assay, but when PR1P was added to a VEGF pellet, the combination stimulated more corneal angiogenesis than VEGF pellets alone, thus confirming that PR1P required VEGF for biological function. Next, we showed that PR1P enhanced angiogenesis following photocoagulation laser treatment-induced injury in a murine choroidal neovascularization assay and enhanced angiogenesis and wound closure in a murine wound-healing model. Finally, treatment with PR1P significantly improved reperfusion in a murine hind-limb ischemia model. Together, our findings support the use of PR1P in vivo to improve outcome from tissue injury by enhancing endogenous VEGF mediated angiogenesis. PR1P is thus a promising novel compound that represents an entirely new class of angiogenic therapeutic for the treatment of vascular occlusive disease and other forms of ischemic tissue injury. In addition, by enhancing endogenous VEGF signaling that is upregulated at sites of tissue injury, PR1P could avoid systemic toxicity seen with other modalities of VEGF therapy.

Materials and methods

Animals

Mice: 6-week old female C57BL/6J mice were purchased from Jackson Laboratory Bar Harbor, ME. Protocols for the in vivo studies were approved by the Institutional Animal Care and Use Committee (IACUC) at Boston Children's Hospital.

prominin-1 and VEGF cellulose peptide arrays

Minimum epitope assignments were based on immunostaining of ABIMED spot peptide arrays prepared at the Massachusetts Institute of Technology (MIT) Biopolymers Facility (Cambridge, MA, USA). Each spot comprised a single 12-mer contiguous peptide, a 3-residue offset was used to cover the entire antigen sequence, and the cellulose-bound peptide membranes were incubated with recombinant human VEGF (R&D systems, Minneapolis, MN, USA) at a final concentration of 0.5 µg/ml for 2 h in T-TBS blocking buffer. After three 10 min washes with T-TBS, anti-hVEGF-A antibody (Neomarkers, Fremont, CA, USA) was added (1 µg/ml) in T-TBS blocking buffer and incubated for 1 h, followed by three more washes. The arrays were then incubated with anti-mouse IgG peroxidase-labeled antibody (1 µg/ml) (Abcam, Boston, MA, USA) in T-TBS blocking buffer for 1 h, followed by three 10-min washes in T-TBS. Analyses of peptide-bound VEGF antibody complexes were performed using chemiluminescence.

Reagents and peptides

The three selected 12-mer peptides and the scrambled form of PR1P were commercially synthesized by Biomatik (Wilmington, DE, USA).

I^{125}-VEGF-binding assay

HMVEC (10^5 cells) were incubated in DMEM containing 20 mM HEPES pH = 7.4 and 0.1% BSA for 1 h on ice in the presence of 12 ng/ml I^{125} -VEGF (PerkinElmer, Waltham, MA, USA) in the presence or absence of PR1P, scrambled peptide or other peptide derivatives (0.2 mg/ml). In addition, HMVEC (10^5 cells) were similarly incubated in separate experiments in DMEM containing 20 mM HEPES pH = 7.4 and 0.1% BSA for 1 h on ice in the presence of increasing concentrations of I^{125}-VEGF. Unbound ligands and peptides were washed with cold binding buffer. Cells were lysed with 0.2 N NaOH, and surface-bound I^{125} -VEGF was determined using a γ-counter (PerkinElmer, Waltham, MA, USA).

Mouse corneal micropocket assay

The mouse corneal micropocket assay was performed as previously described [16]. Briefly, mice were anesthetized using avertin (200 mg/kg IP) and slow-release pellets containing either (a) 160 ng carrier-free recombinant human VEGF (R&D Systems, Minneapolis, MN), (b) 100 µg of PR1P or scrambled peptide, or (c) 160 ng carrier-free recombinant human VEGF with 100 µg of PR1P were implanted into the cornea as described [16]. At 6 days following implantation, mice were euthanized and the area of new blood vessel growth was quantified [16].

Choroidal neovascularization (CNV) assay

C57BL6/J mice were anesthetized with avertin (200 mg/kg) IP), and the pupils were dilated with a mixture of 0.5% tropicamide and 0.5% phenylephrine hydrochloride (Mydrin P; Santen Pharmaceutical, Osaka, Japan). Choroidal lesions (4 per eye around the optic nerve) were induced by laser (power 150 mW) using a slit lamp delivery system with a Nidek photocoagulator (GYC2000, Nidek, Osaka, Japan). Following injury, 2, 20, or 200 µg/0.5 µl/eye PR1P or scrambled peptide was injected into the vitreous. Mice were subsequently euthanized by CO_2 inhalation at 14 days following laser injury, the eyes were enucleated and fixed in 4% paraformaldehyde for 60 min, and the choroids carefully dissected from the eyecup and prepared for tissue sections as we have done in the past [17]. Briefly, 8 radial cuts were made in each section from the edge of the eyecup to the equator. The eyecup was then mounted using Aquamount with the sclera facing down and the choroid facing up. Tissue sections were prepared and then stained for blood vessels using Isolectin GS-IB4 Alexa 594 (Thermo-Fisher Scientific Inc, Waltham, MA, USA), and blood vessel density (CNV area (pixels)) was quantified using fluorescence microscopy and image analysis software (Image J, NIH, Bethesda, MD, USA).

Angiogenesis model of murine ear wound healing

C57BL6/J mice were anesthetized with avertin (200 mg/kg), and the dorsal aspect of the left ear was wounded with a circular 1-mm punch. The wound was treated daily with matrigel (BD Biosciences, San Jose, CA, USA) containing either scrambled peptide (SP) or PR1P (180 µg). Wound neovascularization was assessed 5 days after injury by labeling blood vessels in live mice with FITC-dextran (50 mg/ml IV, Vectors labs, Burlingame, CA, USA) and then immediately euthanizing the animals, and analyzing blood vessel density in the ear using fluorescence microscopy and image analysis software (Image J, NIH, Bethesda, MD, USA).

Hind-limb ischemia model and laser Doppler perfusion imaging (LDPI)

Mice were anesthetized with ketamine (200 mg/kg IP) and xylazine (10 mg/kg IP), the groin was shaved and prepped with iodine, and the proximal right femoral artery ligated using a 6–0 silk suture. Following surgery, mice were randomized to receive either PR1P or scrambled peptide (SP) (10 µg) given either IM (i.e., into ipsilateral thigh) or IP starting on day 0 after surgery and administered every other day until day 12. Laser Doppler perfusion imaging (LDPI, PerimedPeriScan PIM II, Stockholm, Sweden) was used to assess blood flow to lower limbs bilaterally immediately after surgery and on postoperative days 6, 9, and 14. Animals were anesthetized for LPDI on days 6, 9, and 14 using isoflurane and were maintained during anesthesia at 37 °C using a heating pad. Lower extremity scans were captured and an ischemic/non-ischemic limb LDPI blood flow ratio computed for each limb.

Statistical analyses

Student's t test was used for statistical comparisons. Unpaired two-tailed values of $p < 0.05$ were considered to be statistically significant.

Results

PR1P binds VEGF

We recently showed that prominin-1 directly interacted with and potentiated the anti-apoptotic and pro-angiogenic

activities of VEGF in vitro and in vivo [15]. To determine whether small peptides containing amino acid sequences derived from prominin-1 could be used to similarly bind and alter VEGF activity, we designed and manufactured 12-amino acid long peptides containing overlapping sequences from prominin-1 and tested their affinity for binding to VEGF using a cellulose peptide array (Fig. 1a, b). Membranes with transferred short peptides were incubated with VEGF and blotted with anti-VEGF antibody. Among the 360 peptides tested, 3 peptides (Prominin Peptide 1 (PP1), PP2, and PP3, sequences shown in Fig. 1a) displayed the greatest binding of VEGF and were each derived from extracellular regions of prominin-1 (Fig. 1b). In a cell binding study which measured radioactive VEGF binding to human microvascular endothelial cells (HMVECs), we found that PP3 increased VEGF binding (eightfold) to cells compared to PP1, PP2 or a scrambled peptide (SP) containing a random 12-amino acid sequence (Fig. 1c). We henceforth refer to PP3 as prominin-1-binding peptide (PR1P). In separate experiments and to assess the importance of the PR1P sequence in mediating VEGF binding to cells, we rearranged or substituted single amino acids within PR1P's original sequence and compared the ability of these modified peptides to augment VEGF binding to endothelial cells compared to the original PR1P. Figure 1d shows that minor modifications to the amino acid sequence of PR1P dramatically reduced the ability of the altered peptide to enhance VEGF binding to endothelial cells, thus highlighting the importance of the specific sequence within

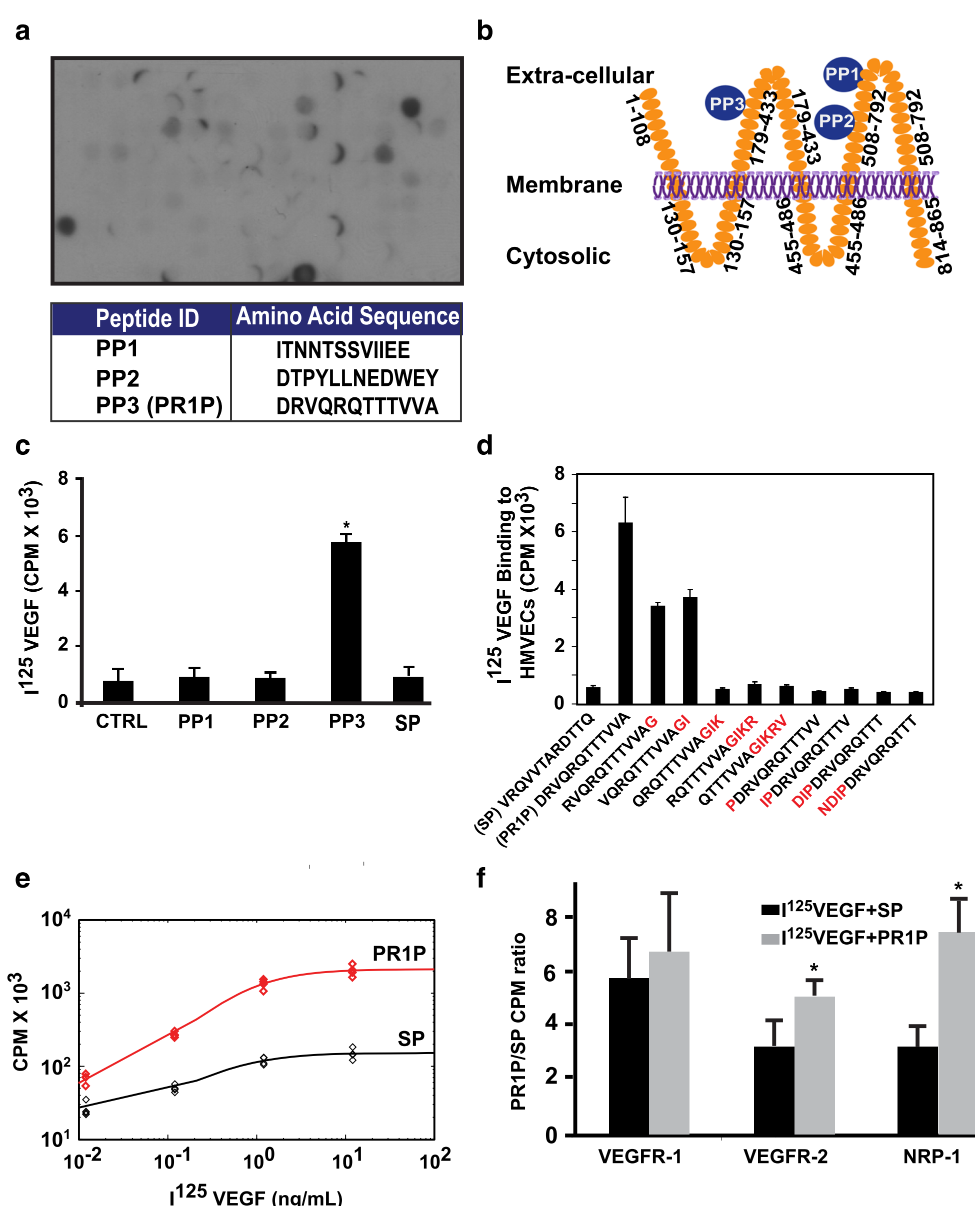

Fig. 1 PR1P enhances VEGF binding to endothelial cells in vitro. **a**–**b** prominin-1 peptide array VEGF-binding assay. Three 12-mer peptides displaying the greatest binding of VEGF were each derived from extracellular regions of prominin-1, as shown in (**b**). **c** Binding of radioactive I^{125}VEGF to HMVECs is increased in the presence of PP3 (PR1P) $P < 0.001$. Control cells (CTRL) were treated with a similar volume of carrier medium as treatment groups. SP (scrambled peptide). **d** Minor modifications including substitutions of single or multiple amino acids (in *red*) in PR1P significantly reduced the effect of modified peptides on binding of radioactive I^{125}VEGF to HMVECs. **e** PR1P increases the binding of VEGF to HMVECs. Plotted are radioactivity levels of cells following incubation with increasing amount of I^{125}-VEGF in the presence of PR1P or SP. Data are mean $\pm$ SEM ($n = 4$). **f** Analysis of ELISA-based radioactive VEGF receptor-binding assay shows that PR1P significantly increased VEGF binding to VEGFR-2 & NRP-1 but not to VEGFR1. Data are mean $\pm$ SEM ($n = 3$) $p < 0.04$. (Color figure online)

PR1P in altering VEGF binding to cells. To further characterize the effect of PR1P on VEGF binding to cells, HMVECs were incubated with increasing concentrations of I^{125}-VEGF in the presence of PR1P or SP, and total binding of radioactive VEGF to the cells was measured (i.e., counts per minute). Figure 1e shows that PR1P increased VEGF binding to the cells by nearly tenfold compared to SP over the ranges of radioactive VEGF concentrations tested. Importantly, these results suggested that PR1P increased the affinity of VEGF binding to VEGF receptors on living cells. Given that VEGF binds several VEGF receptors including VEGFR1, VEGFR-2, and NRP-1 and that VEGF signaling is receptor specific [5], we next used an ELISA-based assay to characterize the effect of PR1P on VEGF binding to specific VEGF receptor subtypes. In this assay, we adhered recombinant VEGFR-1, VEGFR-2, or NRP-1 protein to the surface of 96-well plastic plates and quantified the binding of I^{125}-VEGF to each receptor in the presence or absence of PR1P or SP (Fig. 1f). Interestingly, these studies revealed that PR1P significantly increased VEGF binding to VEGFR-2 and to NRP-1, but not to VEGFR-1 compared to control SP (Fig. 1f).

PR1P promotes angiogenesis in vivo

We recently showed that prominin-1 promotes angiogenesis in vivo [15]. To determine whether the peptide PR1P similarly modulates angiogenesis in vivo in a VEGF-dependent manner, we used a murine corneal micropocket assay where pellets containing VEGF, PR1P, or both were implanted into the cornea of mice. Corneal neovascularization [16] was quantified after 6 days. It should be noted that the cornea does not express significant basal levels of VEGF [18]. PR1P had no effect when delivered alone into the cornea without VEGF (Fig. 2a, b) but enhanced VEGF-dependent blood vessel formation when mixed with VEGF, suggesting that VEGF is required by PR1P for biological function.

To determine whether PR1P upregulated endogenous VEGF-dependent angiogenesis, we quantified new blood vessel development in the presence and absence of PR1P in a murine choroidal neovascularization assay (CNV). In this model, local tissue injury induced by photocoagulation laser treatment increases the expression of VEGF and VEGF-dependent angiogenesis in injured tissue [19]. In our studies, mice received single intravitreal injections of PR1P (2, 20 or 200 μg/eye) or scrambled peptide (SP, 200 μg/eye) after laser-induced injury of the choroid, and angiogenesis at the site of injury was assessed at 14 days. Figure 3a, b shows that PR1P enhanced blood vessel growth following injury in a dose-dependent manner compared to scrambled peptide.

In addition, we also tested the ability of PR1P to enhance wound closure and angiogenesis in a murine wound-healing model. Importantly, normal wound repair is associated with VEGF-dependent blood vessel formation below the wound bed that provides a conduit for nutrients and wound healing [20]. Full-thickness circular holes (wounds) were made in the ears of mice, and the wounds were treated daily with a topical mixture of low growth factor matrigel containing either PR1P or scrambled peptide (180 μg/ear each day for 7 days). PR1P significantly accelerated wound closure compared to scrambled peptide (Fig. 4a–c) and increased the blood vessel density surrounding the wound (Fig. 4a, b, d). Together, these data suggest that PR1P can enhance VEGF-dependent blood vessel formation following tissue injury in vivo.

PR1P improves blood perfusion following ischemic tissue injury

To determine whether PR1P improves blood flow to tissue compromised by poor perfusion, we used a murine hind-limb ischemia model in which we surgically ligated the right femoral artery and treated the animal with PR1P or scrambled peptide (0.4 mg/kg) every other day by local (intramuscular (IM)) or systemic (intraperitoneal, IP) injection. Figure 5a, c shows representative laser Doppler perfusion images (LDPI) of blood flow in the hind limbs of mice at specified time points following surgery and indicated treatment, and Fig. 5b, d shows corresponding ischemic/non-ischemic LDPI blood flow ratios. Note that both local (IM) and systemic therapy (IP) treatment with PR1P significantly improved blood flow to the ischemic limb compared to scrambled peptide.

Discussion

Here we report the design and characterization of a novel 12-amino acid peptide (PR1P) that bound VEGF, enhanced VEGF binding to endothelial cells in vitro, increased angiogenesis in three separate VEGF-dependent angiogenesis models and augmented reperfusion in a hind-limb ischemia model. Importantly, PR1P alone did not induce angiogenesis in the corneal micropocket assay which does not contain endogenous VEGF, but did increase blood vessel formation in the presence of exogenous VEGF, supporting the hypothesis that PR1P is dependent on endogenous VEGF for biological function. In both the choroidal neovascularization and wound-healing models, and in the hind-limb ischemia model, endogenous VEGF levels increase with tissue injury [21–23] and thus our findings suggest that PR1P may serve as a prototype

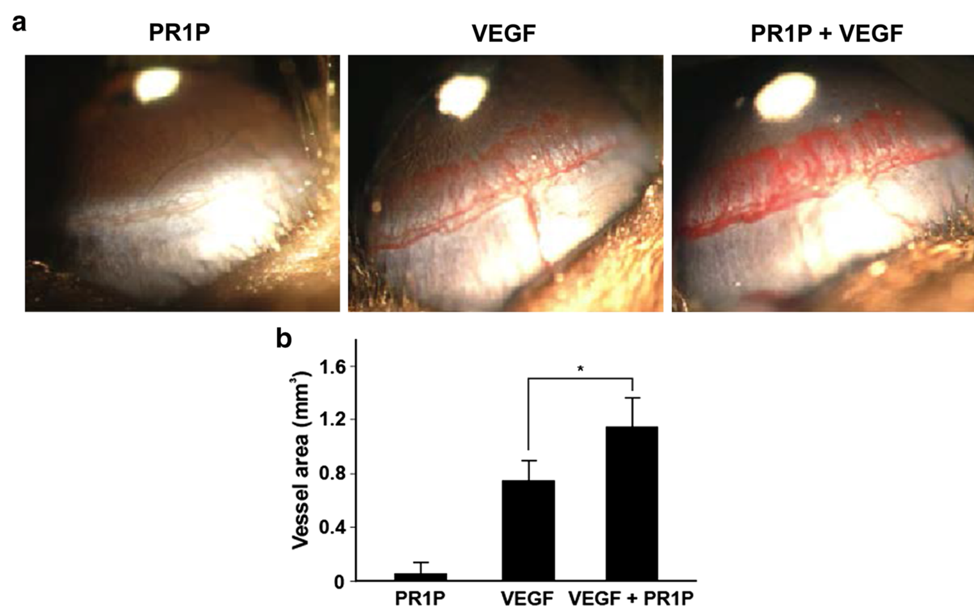

Fig. 2 PR1P enhances VEGF-dependent angiogenesis. Murine corneal micropocket assay. **a** Representative photomicrographs of murine corneas 6 days after implantation of Hydron pellets (0.4 mm^2) containing PR1P (100 μg), VEGF (160 ng), or both, showing enhancement of VEGF-dependent blood vessel ingrowth from the limbus. **b** Histogram shows the corresponding quantification of blood vessel ingrowth described in **a**. Data are expressed as mean ± SD. $*p < 0.01$; $n = 10$

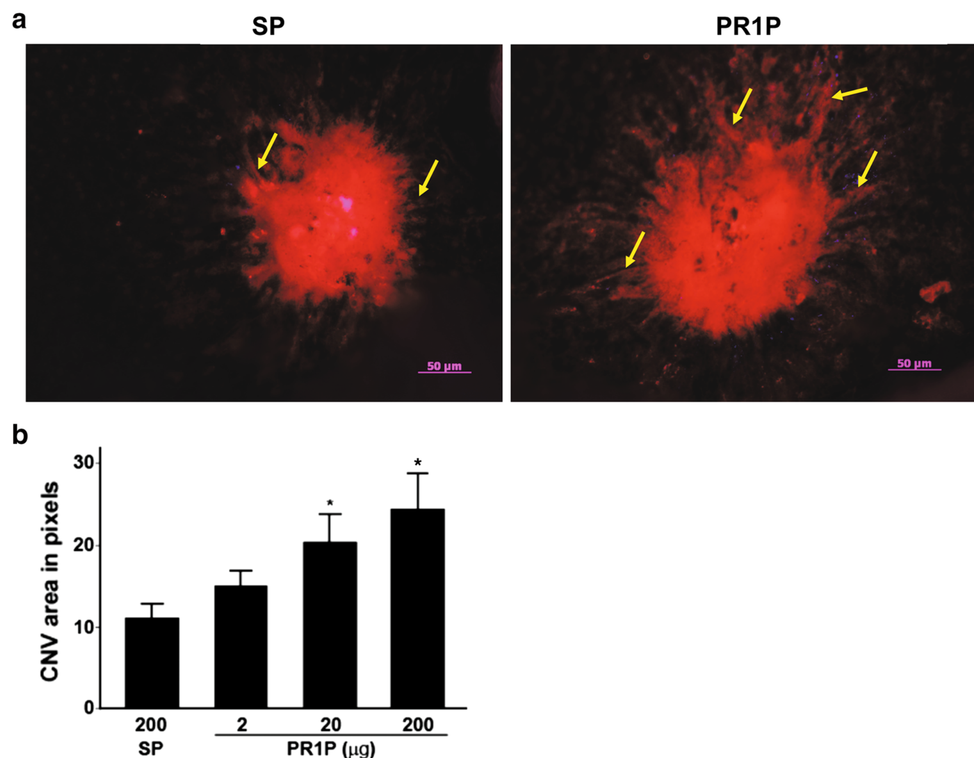

Fig. 3 Choroidal neovascularization (CNV) assay. **a** Representative fluorescence micrographs of choroidal flat mounts showing blood vessel growth 14 days after laser-induced lesions followed by intravitreal treatment with either PR1P or scrambled peptide (SP, 200 μg/eye). Tissue sections were stained with isolectin-IB4 conjugated with Alexa Fluor 594 to identify blood vessels (*red*). *Scale bar*, 100 μm. **b** Histogram shows the quantification of blood vessels in tissue sections from mice as described in (**a**) and treated with intravitreal SP (100 μg) or indicated doses of PR1P. Data are expressed as mean ± SD. $*p < 0.05$; $n = 10$. (Color figure online)

therapeutic agent that can potentiate *endogenous* VEGF and augment its signaling within locally disturbed microenvironments

Angiogenesis, the development of new blood vessels, is vital to the rebuilding and revascularization of tissue, and effective therapies are needed to promote angiogenesis in tissue repair [1]. Targeted or systemic delivery of VEGF isoforms as genes or proteins has been attempted in humans for various clinical conditions including coronary and peripheral artery disease and ischemic ulcers. However, these trials have failed to convincingly demonstrate either significantly improved blood vessel formation in affected injured tissues or sufficient clinical improvement [12]. As a result, VEGF has not received FDA approval for use in humans [13]. The fact that ischemic tissues frequently do not recover despite naturally or experimentally elevated levels of VEGF suggests that the local microenvironment likely plays a significant role in determining

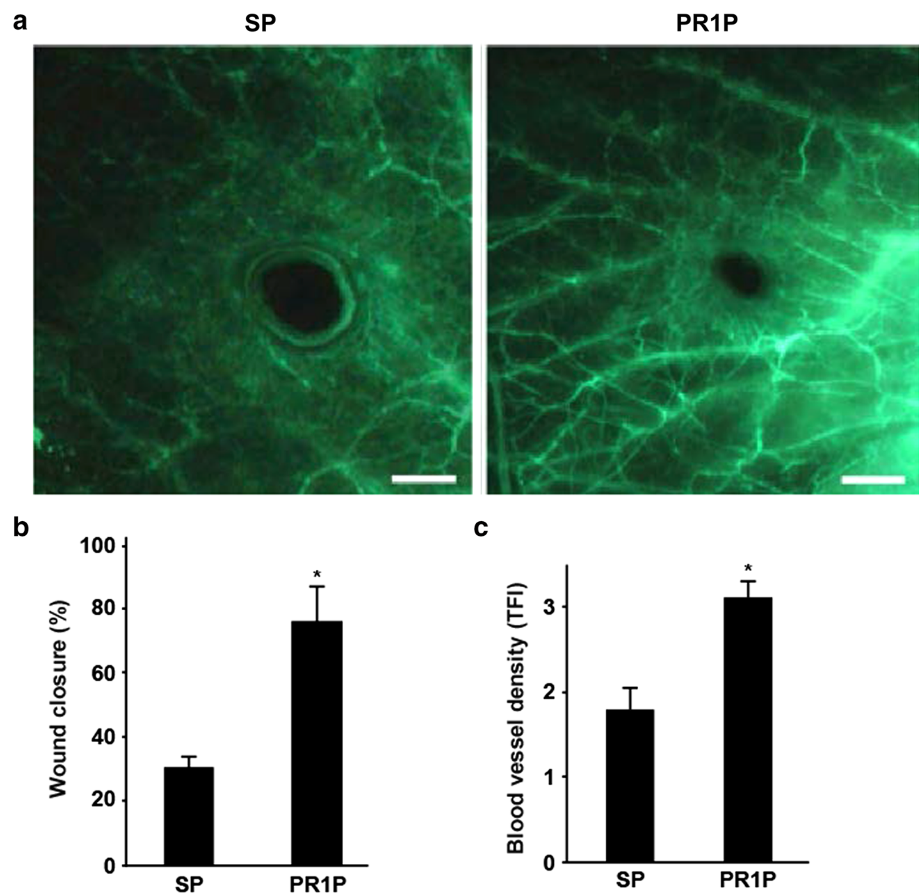

Fig. 4 PR1P accelerates wound closure and angiogenesis in a murine ear wound model. **a** Representative fluorescence micrographs of ears of C56BL6/J mice 5 days after creation of 1-mm circular wounds and daily treatment with matrigel containing PR1P or scrambled peptide (SP, 180 μg). Vessels were visualized by injecting the mice with FITC-dextran. Scale bar, 50 μm. **b–c** Histograms showing corresponding quantification of the percentage of wound closure (**b**) and blood vessel density (total fluorescence intensity, TFI, **c**) from experiments described in (**a**). Note that PR1P treatment enhanced wound closure and blood vessel density. Data are expressed as mean ± SD, $n = 6$, $*p < 0.01$. *Scale bar*, 25 μm

outcome from injury by mediating VEGF signaling. For example, inflammation leads to an increase in proteases known to partially cleave and/or inactivate VEGF in ischemia-induced chronic ulcers of diabetic patients and thus attenuates the effect of increased local VEGF expression [24, 25]. Using three-dimensional computational models of VEGF transport in skeletal muscle, Mac Gabhann et al. surmised that VEGF delivery alone—whether by gene or cell-based delivery—could increase the average activation of VEGFR2 in the tissue, but would not significantly increase the interstitial gradients of VEGF [26]. In addition, they concluded that although VEGF delivery may have increased the activation of VEGFR2, this activation was accompanied by a concomitant increase in the ligation of VEGFR1 which in turn may have modulated VEGFR2 activation. Therefore, VEGF-only therapies may stimulate endothelial activation through VEGFR2, but fail to provide sufficient directional information to guide neovascularization. It is thus noteworthy that we found that PR1P augments VEGF binding to VEGFR2 and to NRP-1 but not to VEGFR1, which may in part explain how PR1P mediates improved outcome in our studies. Thus, alternative therapies aimed at modulating the microenvironment or upregulating endogenous VEGF signaling in VEGF gradients instead of increasing VEGF levels locally or systemically might lead to improved outcome following tissue damage. Results from our studies suggest that PR1P binds endogenous VEGF within damaged tissue and may therefore take advantage of natural VEGF gradients generated within the injured microenvironment to enhance angiogenesis and tissue healing.

Studies using animal hind-limb ischemia models to delineate the many factors influencing the tissue response to ischemia suggest that arteriogenesis predominates in areas proximal to the site of experimental artery ligation [27, 28], whereas muscle necrosis accompanied by an intense inflammatory process, angiogenesis, and muscle regeneration predominates distal to the ligation [27–29]. Although specific mechanisms have not been fully delineated, arteriogenesis is likely induced by fluid shear stress within developing collateral arteries and is not initiated by hypoxia [30]. Inflammation, angiogenesis and skeletal muscle regeneration that occurs distal to the ligation in the presence of tissue necrosis [27–29] is associated with angiogenesis and increased capillary density, especially in areas of severe, acute ischemia [28, 31]. We therefore used the HLI model as an adjunct to the micropocket, wound healing and CNV angiogenesis models to induce

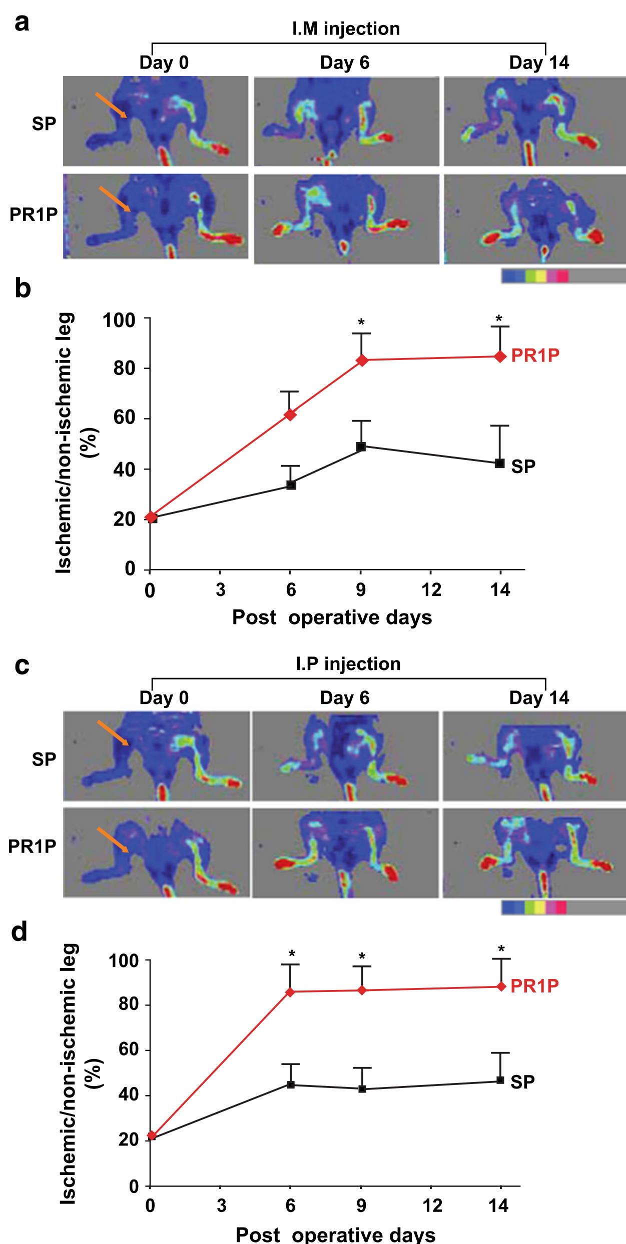

Fig. 5 **a** and **c**) of C57BL6/J mouse hind limbs at indicated time points following right femoral artery ligation and every other day treatment (0.4 mg/kg) by intraperitoneal (IP, **a**) or intramuscular (IM, **c**) injection with PR1P or scrambled peptide (SP). Blood perfusion was quantified using LDPI ratio, i.e., the ratio of average LDPI index of ischemic (*orange arrows*) to non-ischemic hind limb. Colored scale bar represents blood flow velocity in LDPI ratio index. Histograms (**b** and **d**) show the corresponding quantification of the LDPI ratio of the ischemic hind limb at indicated time points from PR1P- and SP-treated animals treated by IM (**b**) and IP (**d**) routes. PR1P treatment (IM and IP) resulted in significantly greater hind-limb perfusion ratios compared to treatment with SP. Data are expressed as mean ± SD, $n = 10$, $*p < 0.001$. (Color figure online)

angiogenesis and compare the efficiency of local vs systemic PR1P therapy in ameliorating recovery from ischemic tissue injury. Interestingly, systemic delivery of PR1P effectively targeted local VEGF gradients induced by artery ligation and tissue ischemia. Although we did not formally evaluate for systemic toxicity, mice appeared to tolerate therapy without adverse effects, and so these studies highlight the promise of a novel approach to target endogenous VEGF gradients at sites of tissue injury without significantly altering normal VEGF signaling elsewhere in the body. Future studies using systemic PR1P therapy will focus on pharmacokinetic profiles and effects on endogenous VEGF signaling in normal and ischemic tissue.

We found that the functional activity of PR1P was highly sensitive to modifications—rearranging or substituting single amino acids within the PR1P sequence near-completely eliminated its ability to enhance VEGF binding to endothelial cells (see Fig. 1d). Interestingly, a specific spontaneous mutation in multiple patients from different families resulting in the replacement of a single amino acid (R to C (R373C)) in prominin-1 that corresponds to the amino acid in PR1P led to an autosomal dominant form of macular dystrophy and degeneration [32]. Affected individuals had increased rates of miscarriage and had dysfunctional endothelial progenitor cells (EPCs) which suggested possible defective angiogenesis [32]. In addition, transgenic mice expressing this R373C mutant human form of prominin-1 displayed defective optical disk morphogenesis [33]. Interestingly, when we substituted the fifth amino acid (R) for C in PR1P, we found that the new peptide containing this substitution no longer retained the ability to upregulate VEGF binding to endothelial cells compared to PR1P. Together, these observations highlight the importance and the evolutionary significance of the amino acid sequence contained in PR1P (and thus in prominin-1) and suggest that prominin-1 binding to VEGF may be altered in patients carrying the prominin-1 R373C mutation. Further studies will be necessary to test this hypothesis and to characterize the molecular determinants enabling VEGF stabilization by either prominin-1 or PR1P in vivo.

In summary, our studies revealed that a novel 12-amino acid peptide (PR1P) derived from an extracellular VEGF-binding domain of prominin-1 bound VEGF, enhanced VEGF binding to endothelial cells in vitro, improved outcome in three separate in vivo models of VEGF-dependent angiogenesis, and improved reperfusion of ischemic limbs following unilateral femoral artery ligation. PR1P required VEGF for biological function and improved outcomes in models where VEGF expression is transiently increased due to tissue injury [23]. Our data provide a proof of principle for a novel approach using a small targeting peptide to bind endogenous VEGF at sites of tissue injury where its expression is naturally upregulated [23]. This approach represents a potential paradigm shift in pro-

angiogenic therapy that could also reduce the deleterious side effects of systemic protein or gene therapy that has plagued the field to date. The strategy of potentiating VEGF or other growth factors with small peptides that enhance factor signaling might also be applied to model diseases in many organs including the lung, heart, and central nervous system.

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