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Expansion of microvascular networks *in vivo* by phthalimide neovascular factor 1 (PNF1)

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Abstract

Phthalimide neovascular factor (PNF1, formerly SC-3-149) is a potent stimulator of proangiogenic signaling pathways in endothelial cells. In this study, we evaluated the *in vivo* effects of sustained PNF1 release to promote ingrowth and expansion of microvascular networks surrounding biomaterial implants. The dorsal skinfold window chamber was used to evaluate the structural remodeling response of the local microvasculature. PNF1 was released from poly(lactic-co-glycolic acid) (PLGA) films, and a transport model was utilized to predict PNF1 penetration into the surrounding tissue. PNF1 significantly expanded microvascular networks within a 2 mm radius from implants after 3 and 7 days by increasing microvessel length density and luminal diameter of local arterioles and venules. Staining of histological sections with CD11b showed enhanced recruitment of circulating white blood cells, including monocytes, which are critical for the process of vessel enlargement through arteriogenesis. As PNF1 has been shown to modulate MT1-MMP, a facilitator of CCL2 dependent leukocyte transmigration, aspects of window chamber experiments were repeated in CCR2^{-/-} (CCL2 receptor) mouse chimeras to more fully explore the critical nature of monocyte recruitment on the therapeutic benefits of PNF1 function *in vivo*.

Keywords

Small molecule delivery; Controlled release; Microvascular remodeling; Monocyte recruitment

1. Introduction

The development of effective strategies to promote vascularization is critical to treatment of a wide variety of diseases and pathologies, including heart disease, peripheral limb ischemia, osteonecrosis and diabetic wounds. To this end, greater research emphasis has been directed in recent years toward an understanding of the complex interplay of many soluble factors, insoluble adhesion molecules within extracellular matrix, and hemodynamic factors that

regulate vascular assembly *in vivo*. Common approaches for therapeutic induction of tissue vascularization have primarily focused on controlled delivery of peptide-based growth factors, such as VEGF and bFGF, which trigger capillary sprouting and remodeling of host microvessels [1,2]. Scaffold biomaterial systems have also been engineered to encapsulate and deliver bioactive growth factors to direct assembly of microvascular networks within regenerating 3D tissues. However, there are several potential drawbacks to protein delivery strategies including high costs to both synthesize and purify recombinant proteins and risks of protein aggregation and degradation [1,3] that are exacerbated during sterilization, solubilization and storage [4]. Additionally, various pathologies associated with ischemic conditions, including hypertension [5], coronary artery disease [6], and atherosclerosis [7–9] often result in endothelial dysfunction [6] and an altered microvascular response to angiogenic growth factors [10]. The refractory nature of these diseased tissues toward endogenous growth factors may limit the utility of these agents as clinical strategies for therapeutic neovascularization.

Recent advances in computer assisted drug discovery and high-throughput phenotype screens of synthetic and natural product libraries have resulted in a variety of non-peptide small molecules that are capable of regulating cell and tissue function. The discovery of new small molecule therapies that specifically promote neovascularization may allow for the creation of functional vascular networks without the cost and classical limitations of protein-based therapies. Our group recently reported the development of a new synthetic small molecule inducer of angiogenesis, phthalimide neovascularization factor (PNF1, formerly known as SC-3-149) [11]. PNF1 is a structural analog of thalidomide with a cyclopentanone substitution on the imide group. *In vitro* evaluation of the compound demonstrated efficacy in enhancing proliferation, survivability, and capillary network formation by endothelial cells. Preliminary *in vivo* studies also indicated an early stage angiogenic response induced by the drug in rat mesenteric windows [11]. Subsequently, network analysis tools were utilized to identify genetic networks of the global biological processes involved in PNF1 stimulation, and to describe known molecular and cellular functions that were most highly regulated by the drug. The most significantly perturbed networks identified gene products associated with transforming growth factor- β (TGF- β) after 24 h, a protein which has many known implications on angiogenesis [12]. Further interrogation of PNF1's mechanism using a novel compendium method identified both tumor necrosis factor- α (TNF- α) and TGF- β as signaling pathways associated with drug function over 1–48 h [13]. These studies suggest that PNF1 and its analogues may serve as promising new angiogenic agents for targeted drug delivery and therapeutic neovascularization.

In this study, we evaluate the *in vivo* therapeutic potential of sustained local delivery of PNF1 to effect vascular remodeling of networks within the dorsal skinfold window chamber model [15–20]. Although capillary sprouting via angiogenesis is currently the dominant focus of proposed strategies for tissue vascularization, diameter increase of resistance vessels through the process of arteriogenesis can also reduce network resistance and better distribute blood flow to tissue areas of ischemic insult. Hence, we evaluate both angiogenic and arteriogenic effects of PNF1 in this model. We also interrogate the role of monocyte recruitment on PNF1-induced microvascular growth and remodeling by quantification of CD11b⁺ cells in tissues treated by the drug. Previous data suggesting PNF1 modulates MT1-MMP expression, a facilitator of CCL2 dependent leukocyte transmigration [21,22], window chamber experiments are replicated using CCR2^{-/-} (CCL2 receptor) mouse chimeras to more fully explore the critical nature of monocyte recruitment on PNF1 function *in vivo*. We believe that the elucidation of the effects of PNF1 on the recruitment of circulating cells and leukocyte transmigration in particular introduces exciting new opportunities for clinical use of the compound for translational biomaterials and tissue engineering research.

2. Materials and methods

2.1. Synthesis of PNF1

As previously described [11], a mixture of potassium phthalimide and 2-chlorocyclopentanone was stirred at 60 °C for 3 h under nitrogen atmosphere. The solution was poured into ice-cold water and a solid was collected by vacuum filtration. The crude solid was purified by column chromatography with 50% hexane/ethyl acetate to yield 15% PNF1 as a white solid (Fig. 1).

2.2. PNF1 encapsulation into PLAGA microspheres and thin films

To fabricate PNF1 or VEGF-loaded microspheres, 50:50 PLAGA (methyl ester capped, inherent viscosity = 0.77 dL/g, Mw = 72.3 kDa; Lakeshore Biomaterials, Birmingham, AL) was dissolved in methylene chloride (Fisher Scientific) at 25% (wt/v), and PNF1 or VEGF (Peirce Endogen, Rockford, IL) was added at appropriate concentrations. The solution was slowly poured into a 1% (wt/vol) polyvinyl alcohol solution stirring at 250 rpm, and the solvent was allowed to evaporate overnight at room temperature, as previously described [23]. The microspheres were collected after 24 h via vacuum filtration, washed extensively with distilled water, and vacuum-dried to remove any remaining solvent.

A solvent casting method was used to fabricate PNF1-loaded 50:50 PLAGA thin films. Specifically, 1.9 g polymer was dissolved in methylene chloride (approximately 8 mL; Fisher Scientific, Hampton, NH); then, 95 mg PNF1 was dissolved into the solvent/polymer liquid, which then cast in a Teflon mold at -20 °C for 1 week and subsequently vacuum-dried to remove residual solvent. Individual 1 mm diameter thin films were cut using a biopsy punch (Acuderm, Inc., Ft. Lauderdale, FL). Before implantation, average film thicknesses were measured using calipers (L.S. Starrett Co., Athol, MA) as follows: 517 ± 41 µm for unloaded PLAGA, 504 ± 29 µm for 5% PNF1. This thickness allowed the films to fit easily into the skinfold chamber without rolling in the space. Additionally, the thin film preparation allowed for 100% incorporation of PNF1.

2.3. *In vitro* migration and proliferation in response to controlled release of PNF1

Human microvascular endothelial cells (HMVEC) (Cambrex, Walkersville, MD) were cultured in EGM2-MV (bulletkit, BioWhittaker, Walkersville, MD) supplemented as directed with 5% fetal bovine serum on tissue culture plates (Nalge Nunc, Rochester, NY) at 37 °C in a humidified chamber with 5% CO₂. To assess cell proliferation, HMVECs were plated subconfluently (1.5×10^4 cells/cm²) in media containing 5% serum and 20 mg of the appropriate microsphere formulation. Cellular proliferation was assessed after 3 and 7 days of culture using an MTS assay (Promega, Madison, WI). To assess cell migration, HMVECs were seeded (200,000 cells/well) into the top of transwell migration chambers (8 µm pores, Corning Costar, Corning, NY) in media containing 5% serum. Ten milligrams of appropriate microsphere formulation was supplied to the bottom of the chamber with cell media containing 5% serum. After 24 h, cells that had migrated to the bottom of the transwell were fixed and stained with crystal violet.

2.4. Quantification of release kinetics and mathematical model of PNF1 release

Qualitative images of PNF1 release from PLAGA films were captured by imaging the fluorescence intensity of loaded films compared with unloaded controls as a function of time. Across 6 weeks, photographs of drug-loaded or unloaded films ($n = 3$) were taken with a 10× objective (Axioskop 40, Carl Zeiss, Germany) using a FITC filter (excitation 450–490 nm, emission 515 nm). Alternatively, PNA (*p*-nitroaniline, FW = 138.12 g/mol) was used as a surrogate small molecule to estimate the initial diffusivity of PNF1 released from PLAGA films. Specifically, 5% PNA was loaded in PLGA according to solvent casting methods above.

Approximately 15 mg of either 1 mm loaded films or 1 mm unloaded films were placed in 2 mL of simulated body fluid (SBF) [49] and incubated at 37 °C. Over 6 weeks, the 2 mL of SBF containing PNA release from films was collected and the absorbance of PNA was quantified on a spectrophotometer at 400 nm. The SBF was refreshed after each collection and results were added over time to yield a cumulative release profile from which the diffusion coefficient could be calculated.

The diffusion coefficient (D_1) for PNF1 release within PLAGA films was calculated by plotting the cumulative release fraction of PNA (i.e. average measure of absorbance) versus the square root of time as previously described by Bezemer et al. [24] using the following equation:

$$\frac{M_f}{M_\infty} = 4 \sqrt{\frac{D_1 t}{\pi l^2}}$$

where M_f/M_∞ is the fractional release from the polymer, t is time, and l is the thickness of the film.

This D_1 was applied to the previously reported mass transport model [28] to describe the dynamics of PNF1 concentration as a function of radial distance. Here:

$$\frac{\partial C}{\partial t} = D_1 \frac{\partial^2 C}{\partial z^2} \quad 0 < z < r_f$$

$$\frac{\partial C}{\partial t} = D_2 \frac{\partial^2 C}{\partial z^2} - k_u C \quad r_f < z$$

where D_2 is the diffusivity within the tissue space ($4.3 \times 10^{-7} \text{ cm}^2/\text{s}$) and k_u is the drug uptake term ($4.4 \times 10^{-4} \text{ s}^{-1}$). These values were assumed based on drugs of a similar molecular weight [25,26] and used in other small molecule models [27]. The concentration model was then solved using mathematical software (MATLAB 7.2, Natick, MA).

2.5. CCR2^{-/-} bone marrow transplant procedure

A donor mouse with a mutation knocking out expression of the CCL2 (MCP-1) receptor, CCR2 (B6.129S4-*Ccr2*^{tm1lf/J}), was used. Host marrow in recipient C57BL/6 mice was eradicated through administration of two 550–600 rad doses of irradiation over 3 h. Donor mice were euthanized with 0.15–0.20 mL pentobarbital sodium. Following local sterilization with ethanol, the donor leg was excised and the femur and tibia were extracted. After dissection of bone epiphyses, cold sterile phosphate-buffered saline was flushed through the marrow cavity to remove marrow cells. After cell disaggregation and resuspension in isotonic NaCl, 3.4×10^6 CCR2^{-/-} marrow cells were injected via tail vein, and the animals were allowed a 2 month recovery.

2.6. Window chamber model used to quantify vessel remodeling *in vivo*

Male C57BL/6 mice (approximately 25 g, Harlan, Indianapolis, IN) were treated with a pre-anesthetic of atropine (0.04–0.4 mg/kg i.p.), and were anesthetized with an i.p. injection of ketamine (60–80 mg/kg) and xylazine (5–10 mg/kg). A circular incision corresponding to the 12 mm chamber diameter was made on one side of the dorsal skin fold, leaving only fascia. Exposed tissue was irrigated with Ringer's solution. Two titanium frames were sutured to opposing sides of the skinfold, a 12 mm glass coverslip (Fisher Scientific) was installed and

the animal was allowed to recover. Following surgery, buprenorphine (0.1–0.2 mg/kg s.c.) was administered as a post-procedural analgesic.

All window chamber tissues were imaged noninvasively. Mice were anesthetized with inhaled isoflurane and their glass windows were removed. Tissues were treated with 0.1 mM adenosine in Ringer's solution to maximally dilate vessels across all timepoints while irrigating the exposed tissue. Film implantation occurred at the first time point (hereafter called "day 0"), seven days after surgical installation of the window chamber. In each window chamber, two unloaded or drug-loaded 1 mm films were implanted. Using a 4× objective on an Axioskop 40 microscope (Carl Zeiss, Germany) adjacent fields of view were captured using a digital camera (Olympus, Center Valley, PA) and were later assembled into one image of the entire window chamber (Adobe Photoshop CS, San Jose, CA). Mathematical modeling in this paper and previously published results from our lab [28] describe the local release profile of small molecules from PLAGA thin films. Specifically, therapeutically relevant concentrations of PNF1 are predicted to extend no further than 2 mm radially from the film's outer edge. Given the dimensions of the window chamber, film placement is such that we do not anticipate overlapping signals between the two implants (Fig. 2).

2.7. Quantitative assessment of vessel remodeling in response to PNF1

Measurements of total microvascular length density and directed ingrowth were used to characterize remodeling of vessel networks in response to locally delivered PNF1. Intravital microscopy images were analyzed using Adobe Photoshop and Image J (National Institutes of Health, Bethesda, MD). After calibration, image areas corresponding to a 2 mm radius surrounding the implanted polymer film were cropped for further analysis. Digitized images were traced and skeletonized to obtain microvascular length density measurements (recorded in vessel length in millimeters per tissue area; $n \geq 13$ films per experimental condition). After tracing, directed ingrowth measurements were performed. Individual vessel tips were analyzed as to whether, when extrapolated, the vector representing vessel tip direction would intersect the edge of the film. Fractal dimensions of traced network images were measured using the boxcounting algorithm in MATLAB v. 7.5 (The MathWorks, Inc., Natick, MA), as described previously [29,30]. Internal diameters were measured via blood column widths at the bisection of each vessel segment between branch points ($n \geq 7$ films per experimental condition).

To prepare window chamber tissue for cyrosectioning and immunostaining, mice were anesthetized with 2% isoflurane mixed with 1 mL/min O₂ and euthanized with i.p. pentobarbital overdose. Immediately, the chest cavity was opened and the vasculature was perfused with 10 mL Tris-buffered saline (TBS) with 0.1 mM CaCl₂ and 2% heparin through the left ventricle, followed by 10 mL TBS solution with CaCl₂, and 10 mL 4% paraformaldehyde (PFA) for vessel fixation. The window chamber tissue was then excised and further fixed in 4% PFA.

Subsequently, window chamber tissue was prepared overnight in 20% sucrose, 7.5% gelatin, and 0.02% sodium azide in PBS. Subsequently, samples were snap-frozen in 2-methylbutane chilled by liquid N₂ and sectioned into 10–20 μ thick sections with a cryotome. Slides were treated with 1:500 Cy3 conjugated monoclonal anti-SMA (IA4 clone, Sigma, Saint Louis, MO) and 1:200 monoclonal anti-CD11b (integrin alpha subunit in Mac-1, clone M1/70.15, Abcam, Cambridge, MA) for 1 h at 20 °C. CD11b antibodies were conjugated with 1:500 Cy3-conjugated IgG secondary antibody (Jackson ImmunoResearch, West Grove, PA). Tissues were then rinsed, mounted, and imaged using five fields of view from the center of each tissue section.

2.8. Statistics

All data are reported using mean $\pm$ SEM, and were analyzed using a one-way ANOVA followed by pairwise comparisons with Tukey's post-hoc tests. Significance was asserted at $p < 0.05$, unless otherwise noted.

3. Results

3.1. Enhanced migration and proliferation in response to PLAGA-encapsulated PNF1

We are interested in releasing PNF1 from biodegradable polymers to treat ischemic diseases and to revascularize tissue-engineered implants. We verified that PNF1 would retain its bioactivity after release from PLAGA matrices, and identified a controlled release formulation of PNF1 in PLAGA that had functional equivalence to that of PNF1 supplemented exogenously at its EC_{max} ($30 \mu M$). Fig. 3A demonstrates significantly increased ($p < 0.05$) HMVEC migration in response to either $30 \mu M$ exogenous PNF1, 0.4% controlled release of PNF1, and 0.005% controlled release of VEGF (concentrations analogous to those presented in literature for stimulation of endothelial cell migration [31]). Additionally, within the limitations of this assay, the response of HMVEC to each of these angiogenic agents was approximately equal.

A proliferation assay was then performed over 14 days in response to control (unloaded), 0.1%, 0.4%, 1%, 2%, and 5% PNF1-loaded microspheres. The 5% PNF1-loaded spheres stimulated significant ($p < 0.05$) HMVEC proliferation after 7 and 14 days (see Fig. 3B), as compared with untreated cultures, control, and all other PNF1 formulations. As shown previously [11], HMVEC proliferation has a bi-phasic response to exogenous PNF1, with an EC_{max} of $30 \mu M$. Similarly, we expect PNF1 films loaded at various concentrations to show a bi-phasic response. Minimal increases in HMVEC proliferation at low PNF1 loadings contrasted to a significant increase at 5% PNF1, confirmed our previous observations and our calculated release formulation, where 5% loading matches closer to the EC_{max} . Therefore, for *in vivo* experiments, it was determined that controlled release formulations of 5% would be utilized.

3.2. Diffusion model of PNF1 release from PLAGA films

The fluorescence of PNF1 encapsulated within PLAGA films appeared inhomogeneous from light microscopy images (Fig. 4A). In addition, the mathematical model of PNF1 release is particularly sensitive to small perturbations in the magnitude of D_1 . Therefore, PNA was used as a surrogate small molecule to estimate the initial diffusivity of PNF1. The experimentally measured value of D_1 ($3.69 \times 10^{-10} \text{ cm}^2/\text{s}$) was calculated by plotting initial fractional release as a function of square root of time using release data of 5% PNA-loaded PLAGA films (Fig. 4B). This value matches within an order of magnitude of previously published values for initial small molecule diffusivity [28,50]. Mathematical modeling predicted that the concentration gradient of PNF1 is confined to $a < 2 \text{ mm}$ radius from the film edge within the tissue space over the 7 day time period (Fig. 4C). Because PNF1 is physically encapsulated within the PLAGA films, but not chemically bound to the polymer chains, the drug release profile is not directly dependent on polymer chain degradation but is predominantly influenced by bulk polymer diffusion kinetics. Complete degradation of 50:50 PLAGA is several weeks long, however, polymer degradation within the first 7 days is enough to release significant amounts of physically entrapped PNF1 for biological effect.

3.3. PNF1 delivery increases vascular length density

Intravital microscopy images of dorsal skinfold tissue were captured at 0, 3, and 7 days post-implantation with unloaded PLAGA films or films loaded with 5% PNF1 to track local changes in microvessel remodeling (Fig. 5A). The smallest observable vessels at $4\times$ magnification are approximately $12 \mu m$ in diameter. The change in length density for 5% PNF1 was >15 -fold

more than for PLAGA after 3 days (see Fig. 5B), and remained significantly increased ($p < 0.001$) over the seven-day course of treatment. Here, increases in microvascular length density was measured as a function of distance from the implant (Fig. 5C). Changes in microvascular length density were measured at distances 0–0.5, 0.5–1.0, 1.0–1.5, and 1.5–2 mm from the drug-loaded film edge. Fig. 3 shows significant increases in length density occurring within the first 1.5 mm of tissue from the edge of the drug carrier.

Notably, the extent of microvessel density increases over unloaded controls was significant at distances nearest to the implant. However, length density measurements increase with decreasing PNF1 concentration and increasing distance from the film. Observations of these trends may result from less accurate estimates for unknown kinetics of PNF1 uptake and degradation and thus create a different release profile. Another possibility is that there are other complex cascades of cell–cell signaling secondary to PNF1 stimulation that control network growth. Furthermore, between a distance of 1 and 1.5 mm, the predicted *in vivo* concentration of PNF1 is approximately 30 μM , the EC_{max} . Therefore, greatest values for length density measurements match well with the model-predicted EC_{max} concentration.

3.4. PNF1-stimulated microvascular network expansion

Implantation of unloaded PLAGA films stimulated an initial *decrease* in arteriolar diameter size after 3 days (Fig. 6A). Though the change in arteriolar diameter of unloaded PLAGA films from day 0 to 3 was not significantly different from the increase in arteriolar diameter from day 0 to 7, this trend may be attributed to transient local acidosis effects on smooth muscle cells and/or an inflammation response leading to early acute angiogenic remodeling. Specifically, Rucker et al. showed that PLAGA scaffolds when implanted into mouse window chambers induced transient activation of leukocytes in postcapillary venules at the border zones of the implant and produced a dense granulation tissue layer containing numerous microvessels [32].

Treatment with 5% PNF1 promoted significant increases in arteriolar diameter 3 days post-implantation, and maintained a greater than 6-fold increase in luminal diameter expansion in arterioles over seven days. This effect was paralleled in venules, where PNF1 also stimulated an approximate 6-fold increase in diameter after 3 and 7 days (Fig. 6B).

Percent changes in arteriole and venule lumen diameter expansion were also measured as a function of distance from the implant (Fig. 6C, D). Changes in vessel size were measured at distances 0–0.5, 0.5–1.0, 1.0–1.5, and 1.5–2 mm from the drug-loaded film edge. Fig. 6C demonstrates that controlled release of PNF1 induced statistically significant increases in arteriolar diameter expansion at positions <0.5, 0.5–1.0, and 1.0–1.5 mm from the drug-loaded films. A similar trend was shown for venules (Fig. 6D), although this data is not significant.

The magnitude of arteriolar diameter changes varied according to the initial vessel diameter. Arterioles with smaller initial diameters underwent significantly greater luminal diameter expansion than larger vessels in the same region of tissues (Fig. 7A, B). A significant effect was seen in the smallest arterioles (<30 μm) after three days, with significant increases over control samples for all arterioles size ranges (<30, 30–50, and >50 μm) after 7 days. PNF1 also promoted significant increases in venules with starting sizes of <50 μm after 3 days, and in venules with 50–150 μm diameter after 7 days (Fig. 7C, D).

3.5. Directed growth of PNF1-stimulated microvascular networks

Quantification of the number of vascular tips visible at 0 and 7 days resulted in a significant increase in the number of vessels in the PNF1-stimulated groups after 7 days (Fig. 8A). Of these vessel tips, the number that when extrapolated would intersect the plane of the film at 0

and 7 days were quantified. Fig. 8B demonstrates the significant increase in the number of such “directed” vessels induced by PNF1 after 7 days. Notably, there was no increase in the total number of vessels or in the number of “directed” vessels in the unloaded film groups.

Normal vascular networks exhibit consistent tree-like branching patterns and constant intravascular distances, while leaky tumor vasculature is much more disorganized [29]. Fractal analysis has been utilized extensively to characterize microvascular patterning [29,30,33], with increases in dilation symmetry, measured by fractal dimension (d), signifying more highly patterned vascular tissues. Traced images of unloaded and 5% PNF1-loaded were subjected to fractal analysis; on “day 0” (before polymer film implantation), dorsal skinfold window chamber tissue exhibited a fractal dimension of $d = 1.63 \pm 0.01$, which is consistent with that measured in the dorsal skinfold window chamber [29,30]. After implantation, tissue stimulated with unloaded PLAGA films demonstrated a constant fractal dimension of $d_{\text{unloaded}} = 1.65 \pm 0.01$ at days 3 and 7, as displayed in Fig. 8C.

Compared with this baseline level of vascular remodeling, release from 5% PNF1-loaded films promoted a significant ($p < 0.05$) increase in fractal dimension to $d_{\text{PNF1}} = 1.68 \pm 0.01$ after the seven-day treatment period. These data demonstrate that while PNF1 stimulates marked microvascular remodeling and expansion, the drug maintains and even significantly increases the regularity of vascular tissue patterning.

3.6. Increased lectin+ and SMA+ staining in PNF1-stimulated vessel networks

Quantification of vascular remodeling via length density measurement is highly valuable for determination of the extent of microvascular expansion stimulated after application of PNF1; however, this metric does not specifically detail the separate angiogenic and arteriogenic effects of the drug. For this analysis, tissue from the dorsal skinfold window chamber was histologically sectioned and stained for endothelial lectin or smooth muscle α -actin (SMA), which are markers of capillaries/sprouts and more mature and nascent vessels, respectively. Numbers of lectin+ or SMA+ positive vessels were counted in fields from these sections; results are displayed in Fig. 9. PNF1 stimulated a statistically significant ($p < 0.05$) increase in the number of lectin+ vessels per tissue area, as compared with samples stimulated with unloaded PLAGA. Additionally, a similar trend was observed for SMA+ vessels.

3.7. Assessment of PNF1-induced monocyte recruitment

We have previously shown that PNF1 is a modulator of MT1-MMP activity in human microvascular endothelial cells [unpublished data]. MT1-MMP is known to facilitate leukocyte transmigration [21] with mediation by CCL2 (also known as monocyte chemoattractant factor-1, MCP-1) [22]. CCR2 is the cellular receptor for CCL2 on leukocytes. Targeted disruption of CCL2/CCR2 through the use of CCR2 chimeric mice has previously been demonstrated to impair arteriogenesis in a murine hindlimb model [34]. In this study, experiments were repeated in tissue of mouse chimeras with bone marrow from a CCR2^{-/-} donor in order to determine the importance of monocyte recruitment PNF1 induced expansion of microvascular networks. As demonstrated in Fig. 5A, B, the previously-observed increase in microvessel length density induced by PNF1 was abolished in CCR2^{-/-} mouse chimeras, suggesting a critical role for monocyte recruitment and promotion of arteriogenic processes on PNF1 function.

Recruitment of monocytes/macrophages to PNF1-stimulated tissue was also quantified. Attenuation of drug-induced microvascular remodeling was observed in CCR2^{-/-} chimeric animals, leading to the hypothesis that vascularization by PNF involves inflammatory cells that are critical for the process of arteriogenesis. The number of CD11b⁺ cells in control and PNF1-stimulated tissue sections were quantified, and normalized per tissue area. PNF1-treated

tissues stimulated an increase in the number of CD11b⁺ cells in tissue sections, and most notably in tissue regions within a 2 mm diameter from the drug-releasing film (Fig. 10A). The effect was attenuated back to control levels outside of the 2 mm window of drug stimulation. Representative images (40× magnification) of CD45 staining in control- and PNF1-treated dorsal skinfold tissues are illustrated in Fig. 10B.

4. Discussion

Tissue growth and regeneration in the adult requires a vascular network that meets the metabolic needs of the repairing tissue. Postnatal neovascularization occurs following tissue injury and remodeling or pathogenesis of conditions such as atherosclerosis or tumorigenesis [35]. However, naturally occurring vascular remodeling is often insufficient for the management of ischemic diseases, including peripheral artery disease, as well as for tissue engineering and regenerative medicine applications in which significant nutritive and metabolic demands have been created [36–42]. Therefore, therapeutic (chemical, cellular, or otherwise) stimulation of vascularization is integral for ischemic disease treatment and integration of tissue-engineered constructs into the adult. Therapeutic strategies that capitalize on the natural processes of angiogenesis and/or arteriogenesis are attractive, as they address treatment of vascular insufficiencies by enhancement of blood flow to tissues [43,44].

The use of medicinal chemistry to synthesize bioactive small molecules, combined with the appropriate design of scaffold biomaterials systems, may ultimately result in the evolution of “designer” drug-release systems that can be tailored for tissue-specific applications. We have previously demonstrated that PNF1, formerly known as SC-3-149, promotes endothelial proliferation, migration, and other cellular processes relevant to angiogenesis *in vitro* [11], and that networks of gene products (including many belonging to TGF- β related pathways) integral to the angiogenic process are upregulated 24 h after application of the drug to endothelial cultures [12]. We are interested in releasing PNF1 from biodegradable polymers for controlled release applications; hence, we verified that PNF1 would retain its bioactivity after release from PLAGA matrices *in vitro*. Indeed, we demonstrated that formulations of PNF1 stimulated endothelial cell migration comparable to that of therapeutically relevant concentrations of VEGF, and that controlled release of 5% PNF1 significantly increased proliferation of HMVEC.

To study the effects of PNF1 on microvascular remodeling *in vivo* we employed the murine dorsal skinfold window chamber, as it permits longitudinal intravital observation of individual microvessel through time. Hence, these data are particularly robust and sensitive because differences in relative length density, directed vessel ingrowth, and vessel diameter can be generated from detailed, repeated observation of specific microvessels as they undergo structural remodeling. Here, we demonstrated that controlled release of 5% PNF1 from PLAGA films stimulated significant microvascular remodeling over 7 days, including noticeable ingrowth of microvessels toward the drug-laden implants. PNF1-loaded films promoted statistically significant increases in microvascular length density, a measure of capillary sprouting, and arteriolar diameter expansion, demonstrating the significant biological effect of the drug. Further validation of network remodeling included immunostaining of window chamber tissue sections, demonstrating an increase in both lectin- and SMA-positive vessels in drug-stimulated samples. We anticipate extending future *in vivo* window chamber studies to 21 days with the intent of assessing long-term patency of vessels stimulated by PNF1.

Due to increasing recognition of its importance in achieving sustained restoration of blood flow to ischemic tissues, emerging evidence highlights the complexity of arteriogenesis. This multilayered process involves endothelial cell activation, recruitment of upstream smooth muscle cells and pericytes to abluminal or outer surface of new microvessels, proliferation of

existing smooth muscle cells, and recruitment of monocytes and macrophages [45,46]. Observations of increased length density and luminal diameter expansions in response to local PNF1 delivery may result from multiple targets along this arteriogenic cascade. Specifically, possible targets may include increasing MCP-1 expression on endothelial cells, increasing TNF- α expression, and/or enhancing proliferation of endothelial and smooth muscle cells. However, our data utilizing CCR2^{-/-} chimeras suggests recruitment of monocytes is critical for PNF1 function. These findings were validated using immunohistochemistry for CD11b, a marker for monocytes/macrophages; PNF1 stimulated increased recruitment of CD11b⁺ cells within the drug-stimulated region of tissue. Importantly, these data parallel the previous results of our novel pathway angiogenic compendium analysis [13], implicating the tumor necrosis factor-alpha (TNF- α) and transforming growth factor-beta (TGF- β) signaling pathways as potential PNF1 mechanism of action, as both are critical for the arteriogenic process [47,48]. Additionally, these data support our previous findings that PNF1 alters activity of membrane type 1 metalloprotease (MT1-MMP) *in vitro* [unpublished data] as MT1-MMP has been recently been demonstrated as a facilitator of leukocyte transmigration. Additionally, since it has been demonstrated recently that MT1-MMP is mediated by CCL2, the chemokine for CCR2, it is possible that the CCR2^{-/-} chimeric animals could not engage in PNF1-stimulated vascular sprouting and angiogenesis due to impaired MT1-MMP signaling.

In addition to observe expansion of microvascular networks in tissues stimulated with PNF1, we also performed initial experiments to determine the regularity and patterning of these newly formed vessels. For example, we showed that ingrowth of capillaries and small arterioles and venules observed after 7 days occurred in a directed manner, toward the drug-laden implant. This effect was attributed to a gradient of PNF1 around the film. Initial experiments were also conducted to determine the extent of normal vascular pattern retention after PNF1 stimulation, measured via fractal dimension analysis. These results demonstrated that PNF1 stimulates marked microvascular remodeling and expansion with significantly increased regularity of vascular tissue patterning.

It is also interesting to note that the most profound arteriogenic effects of the drug were seen on the smallest arterioles in the tissue region of interest. These size-specific changes in vessel diameter could be a result of size-related heterogeneity in cell number, receptor profiles, or ECM protein composition within the vascular bed. In this case, PNF1 signaling may take advantage of vessel heterogeneity to target luminal expansion on smaller vessels. Alternatively, basic principles of scale up limitations may apply. All vessels may be equally capable of responding to local delivery of PNF1, but smaller vessels can more easily generate fifty percent increase in diameter expansion compared with larger vessels. Interestingly, expansion of small arterioles may provide damaged or diseased tissue with locally increased blood flow while decreasing network resistance in the area. Results shown here suggest PNF1 may prove to be an effective agent for targeting size-specific remodeling without over-dilating larger vessels within the tissue space.

Diffusion gradients of bioactive small molecules within specific tissue spaces are difficult to measure directly. Instead, we utilized a mathematical model to generate kinetic profiles *in silico* to predict specific tissue regions where the drug would be most active. Based on model predictions of therapeutically relevant drug concentrations within the tissue space, we focused our measurements of vascular remodeling on regions of interest within a 2 mm radius from the edge of each implant. Statistically increased length density and arteriolar vessel maturation occurred in PNF1-treated vessels situated only within 1.5 mm from the drug-loaded films, demonstrating that effective concentrations of the drug were maintained within this region. In future studies, our model may be extended to other controlled release systems to further examine how altered temporal profiles of PNF1 delivery will regulate vessel remodeling *in vivo*.

5. Conclusion

We created a mathematical model describing release into surrounding tissue using an experimentally derived diffusion coefficient of PNF1 from 50:50 PLAGA. Model-predicted concentrations for 5% loaded films within tissue region of interest (2 mm from the film edge) were comparable to those previously shown to be maximally effective in stimulation of microvascular endothelial cells *in vitro*. Delivery of PNF1 at 5% loading was examined in the dorsal skinfold window chamber. Our results show that structural microvascular network remodeling induced by PNF1 is both angiogenic and arteriogenic. Specifically, controlled release of this drug led to spatial and temporal increases in location-dependent vessel density and microvessel diameter in treated tissues, with maintenance of functional vascular patterns. These experiments suggest that PNF1 is effective for therapeutic induction of neovascularization for possible treatment of ischemic tissue disorders and vascularization of engineered tissues.

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Fig. 1.
Synthesis of PNF1 (formerly known as SC-3-149).

Fig. 2. Dorsal skinfold window chamber. One millimeter PLAGA films, either unloaded or PNF1-loaded, are placed within the window chamber. Repeated measurements of the same vessel networks are recorded across 7 days using montaged images from individual light micrographs.

Fig. 3.

Measures of *in vitro* bioactivity of PNF1 after encapsulation in PLAGA. (A) A transwell migration assay demonstrating significant ($p < 0.05$) migration in response to 30 μ M exogenous PNF1, 0.4% PNF1 released from PLAGA microspheres, and 0.005% VEGF released from PLAGA microspheres ($n = 3$). Within the limits of this assay, the migration responses to each of these concentrations of angiogenic factor are approximately equal. (B) HMVEC proliferation in response to unloaded (control), 0.1, 0.4, 1, 2 and 5% PNF1-loaded microspheres ($n = 9$). 5% PNF1-loaded spheres stimulated significant ($p < 0.05$) proliferation, as compared with untreated cultures, control, and all other PNF1 formulations, after 7 and 14 days.

Fig. 4.

Release of PNF1 in a 5% (wt/wt) ratio from PLAGA films. (A) Control and 5% PNF1-loaded 1 mm PLAGA films, as photographed (10× objective) using a FITC filter. The autofluorescence of PNF1 is evident. (B) Cumulative release profile of 5% PNA from 1 mm PLAGA films over the course of 6 weeks ($n = 3$, $\pm$ SEM). Initial diffusivity of the model small molecule from the PLAGA films was calculated as $3.69 \times 10^{-10} \text{ cm}^2/\text{s}$. (C) Drug concentration distribution as predicted by the mathematical model. The distribution profile is modeled for $t = 3$ and 7 days. For both timepoints, the concentration profiles drop off exponentially within a 2 mm radius from the film.

Fig. 5.

Microvascular remodeling in response to PNF1. (A) Intravital microscopy images of blank PLAGA or 5% PNF1-loaded films in wild type or mouse chimeras with one marrow donation from CCR2^{-/-} animals in the dorsal skinfold window chamber 0, 3, and 7 days post-implantation (scale bar = 500 μ m). Substantial microvascular remodeling and arteriogenesis are induced by PNF1 in the wild type animals after 7 days, observable through increased sprouting and luminal expansion of existing vessels at $t = 3$ and 7 days (highlighted by black arrows). Microvascular growth toward the drug-loaded film is evident over the course of treatment. Quantified changes in microvascular length density with 5% (wt/wt) PNF1 films for 7 days are shown in (B). Drug release enhances vascular length density significantly over the course of 0–3 and 0–7 days (* $p < 0.05$, ** $p < 0.001$). This potent effect is attenuated CCR2^{-/-} chimeric mice, demonstrating the necessity of CCL2 (MCP-1) signaling and monocyte recruitment on the function of PNF1. (C) Alterations in length density of vessel segments located <0.5, 0.5–1.0, and 1.0–1.5 mm from the PLAGA film. Delivery of 5% PNF1 initiates significant increases in length density in tissue regions within a 1.5 mm radius from the film (* $p < 0.05$).

Fig. 6. Characterization of the PNF1-stimulated expansion of microvascular networks. (A) Quantification of the total number of vascular tips visible at 0 and 7 days, resulted in a significant increase ($p < 0.05$) in the number of vessels in the PNF1-stimulated groups after 7 days. Of these vessel tips, the number that, when extrapolated, would intersect the plane of the film at 0 and 7 days (called “directed” vessels) were quantified in (B). The number of such directed vessels significantly increased ($p < 0.05$) after PNF1 stimulation for 7 days. Notably, there was no increase in the total number of vessels or in the number of “directed” vessels in the unloaded film groups. (C) Results of a fractal analysis, which is known to provide information on the formative process of vascular networks. Before implantation, vascular

networks in the dorsal skinfold window chamber were characterized by a fractal dimension of $d = 1.63 \pm 0.01$. Unloaded PLAGA-stimulated vascular networks maintained this fractal dimension over the 7-day course of treatment, with a final $d_{\text{unloaded}} = 1.65 \pm 0.01$. Notably, the fractal dimension of tissues stimulated with 5% PNF1-loaded PLAGA films significantly increased to $d_{\text{PNF1}} = 1.68 \pm 0.01$ ($^*p < 0.05$) after 7 days, demonstrating that while PNF1 stimulates marked microvascular remodeling and expansion, the drug maintains and even significantly increases the regularity of vascular tissue patterning.

Fig. 7.

Arteriolar and venular luminal expansion after PNF1 stimulation. Changes in the diameter of arterioles (A) and venules (B) over the course of 0–3 days, or the full treatment period of 0–7 days after implantation with 1 mm PLAGA control films or those treated with 5% (wt/wt) PNF1 films. PNF1 stimulates significantly increased lumen expansion of both arterioles and venules over the course of 0–3 days, as well as over the entire treatment window of 7 days (* $p < 0.05$). Changes in arteriolar and venular diameter as a function of distance from the unloaded or drug-loaded film were characterized (C/D). Release of PNF1 stimulated significantly increased arteriolar diameters in vessels located <0.5, 0.5–1.0, and 1.0–1.5 mm

from the film ($*p < 0.05$). A similar trend was observed for venules, although this result was not significant.

Fig. 8.

Luminal diameter expansion as a function of starting vessel diameter. Changes in average arteriolar (A/B) and venular (C/D) diameter with increasing initial starting inner diameter over a treatment period of 0–3 days (A/C) or 0–7 days (B/D) in a 2 mm radius from an unloaded PLAGA films or those loaded with 5% PNF1. A trend of increasing arteriolar luminal expansion is increased with decreasing initial diameter. Significant increases in average arteriolar diameter were demonstrated for arterioles <30, 30–50, or >50 μm after the seven-day treatment with 5% PNF1 (as compared with control PLAGA films; * $p < 0.05$). A similar trend was also demonstrated in venules of increasing starting diameter.

Fig. 9. Measurement of PNF1-stimulated vascular network expansion by tissue immunohistochemistry. PNF1 released from PLAGA films induced suggestive increases in the number of smooth muscle α -actin (SMA) positive vessels per tissue area. Both results are illustrated in representative images (60 $\times$ magnification; scale bar = 20 μ m) of tissues immunostained for SMA.

Fig. 10.

Recruitment of CD11b⁺ cells to the site of PNF1-induced vascular network expansion.

Quantification of tissue area-normalized CD11b⁺ cells (a monocyte-macrophage marker) in control- and PNF1-stimulated tissue sections (A). PNF1-treated tissues stimulated an increase in the number of CD11b⁺ cells in both entire tissue sections, as well as in tissue regions within a 2 mm diameter from drug-releasing film placement. Notably, the effect was attenuated back to control levels outside of the 2 mm window of drug stimulation. Representative images (40 $\times$; scale bar = 50 μ m) of CD11b staining in control- and PNF1-treated dorsal skinfold tissues are illustrated in (B).