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Sustained release formulations of rhVEGF$_{165}$ produce a durable response in a murine model of peripheral angiogenesis

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ABSTRACT

Local delivery of therapeutic angiogenic agents that stimulate blood vessel formation represents a promising strategy for the treatment of peripheral vascular disease (PVD). At present, requirements for temporal and spatial parameters for localized delivery are unclear, with a variety of sustained delivery approaches being examined. Two polymer-based sustained formulations containing the 165 amino acid isoform of human recombinant Vascular Endothelial Growth Factor-A (rhVEGF$_{165}$) were evaluated for their potential application in the treatment of PVD following intramuscular injection. Microspheres prepared from a 50:50 ratio of polylactic-co-glycolic acid (PLGA) and a gel of PLGA polymer solubilized in N-methyl pyrrolidone (PLGA:NMP) were each loaded with rhVEGF$_{165}$ and tested *in vitro* and *in vivo*. PLGA microspheres averaged ~30 μm in diameter and contained 8.9% (w/w) rhVEGF$_{165}$ while the PLGA:NMP gel was formulated with varying amounts of spray freeze dried rhVEGF$_{165}$ to result in final gel formulations having concentrations of 0.36, 0.72, or 3.6 mg/mL rhVEGF$_{165}$. *In vitro* release of rhVEGF$_{165}$ from PLGA microspheres showed ~10% cumulative release by day 6 whereas the cumulative release of rhVEGF$_{165}$ from the PLGA:NMP gel matrices (0.65% w/w loading) was less than 0.25% at this same time point. While the *in vitro* release characteristics of these two sustained-release formulations were broadly different, the plasma rhVEGF$_{165}$ concentration-time profiles following hind-limb intramuscular (IM) injection of these formulations in non-compromised rats revealed similar *in vivo* pharmacokinetics. Three-dimensional resin casts of vascular architecture were prepared at days 3, 7, 14, 21, 28, 60 and 75 following a single IM dosing of these sustained release microsphere and gel matrix formulations in the gastrocnemius muscle of immune-

compromised mice. Scanning electron microscopic visualization of these vascular casts demonstrated spatial arrangement of capillary sprouts and vessel enlargement consistent with profound vascular changes occurring within three days of dosing that persisted for two months, approximately one month beyond the anticipated completion of rhVEGF$_{165}$ release from these sustained delivery formulations. Vascular re-modeling events were correlated with histological and immunohistochemical parameters attributed to known biological actions of rhVEGF$_{165}$ signaling. Together, these pharmacokinetic and pharmacodynamic results support the use of sustained release PLGA-based formulations for the local delivery of rhVEGF$_{165}$ to achieve a durable vascular re-modeling response.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is a potent angiogenic mitogen [1]. VEGF expression is up-regulated by hypoxia-inducible factor and functions to increase blood vessel diameter by stimulating nitric oxide synthetase, generating the vaso-active agent nitric oxide [2]. Acting through this mediator, VEGF increases vessel wall permeability, creating a pathway for proliferating and migrating endothelial cells to establish nascent blood vessels, as well as inducing local vascular hypotension. VEGF has also been shown to be a pleuripotent cytokine capable of not only inducing endothelial responses but also of recruiting cells such as monocytes that secrete a plethora of additional complementary growth factors [3]. Such potent actions resulting in neovascularization make VEGF a promising therapeutic candidate molecule for the treatment of conditions of compromised vascular health, such as peripheral vascular disease (PVD).

Neovascularization involves several steps: endothelial proliferation that generates nascent vascular buds, directional migration of vascular buds that follows chemotactic growth factor gradients, maturation of the nascent vessels and finally, pruning of non-essential vessels [4]. Previous studies have suggested that VEGF is one of the most potent factors capable of stimulating these events [3; 5]; rapid systemic introduction of a solution containing rhVEGF$_{165}$, however, can result in severe hypotensive shock [6; 7]. While local injection of liquid formulations of rhVEGF$_{165}$ may induce neovascularization, this outcome is transient [8; 9]. Controlled release of liquid rhVEGF$_{165}$ to result in a constant low dose of the molecule by implanted osmotic pumps, however, was found to result in neovascularization and vascular perfusion in a rabbit model of partial limb ischemia [10].

The presence, and thus detection, of rhVEGF$_{165}$ in the systemic circulation after being released from a bioerodible sustained release delivery system is influenced by the capacity of VEGF to bind heparin structures of the extracellular matrix elements in the region proximal to the delivery system [11]. Studies have demonstrated that heparin-binding interactions have significant impact on the pharmacokinetics, and thus potentially the pharmacodynamic properties, of rhVEGF$_{165}$ [12]. The pharmacokinetics of rhVEGF$_{165}$ have been found to be non-linear; rhVEGF$_{165}$ must first saturate the heparin-binding sites before rhVEGF$_{165}$ may be measured in the systemic circulation [12], potentially complicating the ability of the researcher to understand the *in vivo* release characteristics of a bioerodible depot releasing rhVEGF$_{165}$. We have previously examined the nature of these interactions and the fate of VEGF at the site of injection [13].

To date, little clinical success has been achieved using VEGF for the therapeutic treatment of PVD [14]. Since the sustained presence of VEGF appear to be essential for nascent vessel formation and maturation [1; 15], efforts have been made to achieve a durable neovascularization outcome by using local, sustained delivery approaches. Polymer scaffolds containing VEGF and platelet-derived growth factor [16] have shown *in vivo* benefits for the formation of mature new blood vessels. Scaffolds made of poly(ether)urethane-polydimethylsiloxane (PEtU-PDMS) semi-interpenetrating polymeric network (semi-IPN) and fibrin loaded with VEGF and basic fibroblast growth factor have also shown promising results [17]. Collagen scaffolds chemically conjugated with VEGF have also been described [18]. Similarly, scaffolds prepared using an 85:15 ratio of PLGA to deliver rhVEGF$_{165}$ [19] and porous sponges composed of PLGA containing absorb rhVEGF$_{165}$ stimulate new vascular growth following implantation [20].

More recently, microspheres prepared from PLGA [21; 22; 23] as well as PLGA/alginate [24] have been described for the sustained delivery of VEGF. Our interest has also been to identify an injectable format for the sustained delivery of rhVEGF$_{165}$; we have examined the use of microspheres prepared with 50:50 ratio of PLGA to encapsulate rhVEGF$_{165}$ in order to result in its sustained delivery at a local site following injection [25]. One potential concern with the use of PLGA microspheres, however, it their capacity to incite tissue responses [26; 27; 28], events that might compromise rhVEGF$_{165}$ fate and function.

We have now extended those previous studies using 50:50 lactide/glycolide ratio of PLGA microspheres to locally deliver rhVEGF$_{165}$ by comparing that delivery system with PLGA dissolved in a bio-compatible organic solvent, N-Methyl-2-pyrrolidone (NMP) to form a gel that after injection and solvent dispersion will form a single implant that solidifies. While both methods provide a delivery system that can be injected selectively into sites of reduced vascular function, the gel-based system may have pharmaceutical advantages of preparation and handling.

In order to adequately assess relevant outcomes for PVD therapy using these two formulation approaches, methods were established to not only define critical parameters associated with the delivery of biologically active rhVEGF$_{165}$ from these formulations, but also to describe the actions of long-term locally delivered rhVEGF$_{165}$ on vascular re-modeling. Vascular casting was identified as a means to visualize vascular architecture with a high degree of resolution, with minimal artifacts and in three dimensions that also allows for temporal assessment of pharmacodynamic responses [29; 30]. Indeed, this technique has been used to evaluate the effects of a single intramuscular (IM) injection of

liquid VEGF in an induced ischemic animal model [31]. We have correlated vascular casting information with routine histology, up-regulation of cell surface markers present on nascent endothelial cells, as well as *in vitro* and *in vivo* profiles of rhVEGF$_{165}$ release from these formulations. Overall, our data supports the use of either approach, either PLGA microspheres or a PLGA/NMP gel for the local delivery of rhVEGF$_{165}$ by injection to sites of peripheral ischemia to achieve sustained release of this potent mitogen in a manner previously suggested to achieve durable benefits in the context of PVD.

MATERIALS AND METHODS

Materials

E. coli-derived rhVEGF$_{165}$ homo-dimer was produced and purified at Genentech, Inc. (S. San Francisco, CA). The protein was formulated in succinate buffer at pH 5.0 with trehalose and polysorbate 20, then spray-freeze dried to yield a free-flowing solid phase material. Poly(D,L-lactide-co-glycolide acid) (PLGA) as a 50:50 lactide/glycolide ratio with free acid end groups (RG502H) was purchased from Boehringer Ingelheim. N-methyl pyrrolidone (NMP, Pharmasolve, USP grade) was purchased from ISP Technologies and combined with the 50:50 (w/w) lactide/glycolide PLGA as described previously [32]. Sprague-Dawley rats and nude CD-1 immune-compromised mice were obtained from Charles River (Hollister, CA).

Methods

rhVEGF$_{165}$ microsphere preparation

Microspheres ranging in size between 20-90 μm diameter with an average of ~30 μm were prepared as previously described [25]. In brief, spray freeze-dried rhVEGF$_{165}$ powder (prepared as described earlier) was added to a mixture of PLGA/ethyl acetate (1.7 g/ml) for 9% protein loading. The solid phase protein was suspended in the liquid phase by homogenization and the suspension sprayed into a frozen bed of ethanol, subsequently warmed for 48 hours to 70^{o}C to remove the ethyl acetate from the microspheres. After filtration to remove the ethanol, microspheres were dried for 72 hours under nitrogen at 5^{o}C. rhVEGF$_{165}$ loading levels were verified by protein analysis [25].

PLGA:NMP gel preparation

The gel was prepared by adding equal masses of PLGA and NMP to a sterile, capped glass vial and stirred overnight at room temperature to dissolve the polymer. Gel was added by positive displacement pipette to pre-weighed spray freeze-dried rhVEGF$_{165}$, prepared as described earlier [25] in differing masses to provide doses of 3.6 mg/kg, 0.72 mg/kg and 0.36 mg/kg in the same dose volume. Mixtures of spray freeze-dried rhVEGF$_{165}$ and PLGA:NMP gel were made by homogenization at 8,000 RPM for two minutes with a VirTis Cyclone homogenizer (Gardiner, NY, USA).

In vitro release studies

In vitro release studies were performed at 37^{o}C in a release media containing 10 mM histidine, 140 mM NaCl, 0.02% polysorbate 20 and 0.02% NaN_3 at pH 7.0 with buffer replacement at sampling times. The PLGA microsphere formulation contained 10 mg/mL rhVEGF$_{165}$, 1 mg/mL trehalose, 0.01% polysorbate 20, and 5 mM succinate at pH 5.0. The NMP:PLGA (50:50 w/w) formulation contained 10 mg/mL rhVEGF$_{165}$, 10 mg/mL trehalose, 0.03% polysorbate 80, and 10 mM histidine at pH 6.0. The activity of released rhVEGF$_{165}$ was assessed using an enzyme-linked immunosorbant assay (ELISA) assay format to assess VEGF receptor (KDR) binding [25]. As a surrogate assay for potency, this assay used a KDR-IgG chimera construct to demonstrate that rhVEGF$_{165}$ binding to its receptor remained intact. Previous studies showed that rhVEGF$_{165}$ characterized as potent in this assay correlated with rhVEGF$_{165}$ in a dimeric state (shown by size exclusion chromatography) and with the capability of heparin binding (shown by affinity chromatography) [25].

In vivo studies

All *in vivo* experiments were carried out with local Institutional Animal Care and Use Committee (IACUC) approval in accordance with the institution's ethical guidelines.

In vivo pharmacokinetic studies

As VEGF receptors are present on a variety of circulating white blood cells, in particular platelets [4], systemic time-concentration profiles were established by analysis of plasma rather than serum. In order to examine *in vivo* rhVEGF$_{165}$ release and presence in the systemic circulation, formulations containing the highest rhVEGF$_{165}$ concentration, 3.6 mg/kg, were used. At this higher dose, the amount of rhVEGF$_{165}$ released by each depot would overwhelm local capture through heparin binding events in the tissues and allow earlier detection in plasma over time. Normal female Sprague-Dawley rats (200-250 g) were injected in the hind limb gastrocnemius muscle. Rats were used for this aspect of the study rather than mice so that serial blood samples might be taken. Plasma was harvested from whole blood samples taken over a seven-day period. The study was terminated at seven days as prior investigations (data not shown) have shown the production of antibodies to this foreign (human) protein by one week to ten days after dosing. Plasma concentrations of human RHVEGF165 were quantified using a dual monoclonal antibody-based ELISA format that had a limit of detection in plasma of 1 pg/mL [12].

In vivo dose administration

These studies were designed to assess durable responses to rhVEGF$_{165}$ administered in a sustained manner. Female nude CD-1 mice (20-25 g) were used as the animal model because they lack the ability to raise potent immunity to a non-self protein. Mice were injected in the gastrocnemius muscle with rhVEGF$_{165}$-containing or control preparations

of microsphere or gel formulations. rhVEGF$_{165}$ loaded microspheres or PLGA:NMP gels, designed to deliver rhVEGF$_{165}$ in doses of 3.6, 0.72, or 0.36 mg RHVEGF165/kg, were injected using a 23-gauge needle. Additionally, ethylene-oxide sterilized fluorescently labeled polystyrene divinylbenzene beads (nominal diameter of 30 μm, Duke Scientific, Palo Alto CA) were added to each formulation just prior to injection. Unlike the PLGA microspheres, these marker beads did not dissolve during the KOH maceration step (see below) and remained in the tissues. The fluorescent nature of these marker beads provided a method to assess the tissue distribution of a formulation many days after injection. The consistent diameter of these marker beads were used as a microscopic sizing standard when examining vascular casts by SEM. Animals were sacrificed on day 3, 7, 14, 21, 28 and between 60 and 75 days after a single dosing with one of the placebo or active sustained release formulations.

Vascular casting

On the day of sacrifice, heparinized mice were perfused through the descending aorta with phosphate-buffered saline (PBS) under anesthesia. After the blood was exchanged with PBS as completely as possible, the vasculature was perfused with a low-viscosity resin, Mercox acrylic casting material, that consists of a resin (Ladd Research, Williston, VT, USA), to which benzoyl peroxide was added as a catalyst to polymerize the perfused polymer *in situ* at ambient temperature. Both hind limbs were excised after curing for four hours, the skin removed and the muscle tissue of the leg macerated by serial digestions with 7% KOH so that only bone and resin casts remained. Tissue-denuded vascular casts were rinsed, submerged in distilled water, frozen and lyophilized to

maintain the three dimensional structure of the vascular casts. The resulting casts were sputter-coated with gold-palladium and viewed with a Philips XL30 environmental scanning electron microscope.

Vasculature casts of rhVEGF$_{165}$-dosed legs were compared to vasculature casts of untreated contra-lateral legs to account for the potential for systemic exposure as well as localized administration. Because systemic exposure cannot be ruled out and the effect of the delivery system itself might have an effect on the vasculature, control mice were dosed with placebo microspheres and gel to compare the effect of the delivery platform itself.

RESULTS

In vitro *release characteristics of rhVEGF$_{165}$ from gel and microsphere formulations are different*.

Cumulative *in vitro* release profiles for similar concentrations of rhVEGF$_{165}$ formulated in either PLGA microspheres or PLGA:NMP gel for a six-day period are shown in Figure 1. The cumulative release profile for rhVEGF$_{165}$-loaded microspheres demonstrated an initial rapid release, followed by a slower release rate. rhVEGF$_{165}$ release from similarly prepared PLGA microspheres was determined previously and shown to be complete between 25 and 30 days in the same *in vitro* release test system [25] In contrast, the PLGA:NMP gel formulation tested under the same *in vitro* release test conditions released rhVEGF$_{165}$ at an extremely slow rate over the same duration. These differences in release profiles are consistent with these two formulation matrices having different diffusion paths and degradation mechanisms [25; 32].

In vivo *release characteristics of gel and microsphere formulations are similar.*

In contrast to *in vitro* release studies, peak plasma concentrations and total areas under the curve of rhVEGF$_{165}$ following IM injection in normal rats were similar for the sustained delivery formulations prepared using PLGA microspheres and PLGA:NMP gel (Fig. 2). One important difference was observed between these two formulations however. While both formulations show sustained plasma rhVEGF$_{165}$ concentrations over 7 days, the plasma concentration versus time profiles for rhVEGF$_{165}$-containing PLGA microspheres were biphasic whereas the rhVEGF$_{165}$-PLGA:NMP gel formulations demonstrated a profile with a sustained rise and subsequent fall. Plasma concentration-time profiles observed in these studies using PLGA microspheres were considered typical

for this type of formulation: an initial burst resulting in high rhVEGF$_{165}$ plasma levels within the first six hours post dosing, followed by a second peak that was reached a maximum at day 4. Initial peak plasma concentrations of rhVEGF$_{165}$ were likely due to release of readily available protein at the microsphere surface that entered the circulation after saturating tissue (heparin-like) binding sites at the injection site. The second observed rhVEGF$_{165}$ peak, occurring 48 hr after administration, was presumed to be due to rhVEGF$_{165}$ that diffused from the microsphere core and was released by bulk erosion of the polymer matrix.

Plasma concentration-time profiles obtained using normal rats dosed with rhVEGF$_{165}$ loaded PLGA:NMP gels showed a steady rise over the first 4 days that was maintained to day 7, the last day of blood sampling, although there was a transient decline observed in plasma rhVEGF$_{165}$ levels measured on day 5. This type of plasma rhVEGF$_{165}$ profile suggested a slower initial release of rhVEGF$_{165}$ from the PLGA:NMP gel matrix compared to the PLGA microspheres used in these studies. Unlike the PLGA microspheres that produced high plasma levels within 6 hours of IM dosing, the slower rate of plasma level increase supports the hypothesis that rhVEGF$_{165}$ is released from this gel matrix more slowly. These plasma concentration-time profiles reflect systemic rhVEGF$_{165}$ plasma concentrations resulting from the release from the formulation and the saturation of the local tissue binding, which serves as a physiological depot that is considered to be physiologically important for this heparin-binding protein. Such tissue interactions have been previously described and visualized for rhVEGF$_{165}$ at an IM site post injection [13].

Analyses of vascular casts demonstrate localized markers of neovascular events.

Vascular casts prepared from mouse hind limbs dosed with either PLGA microsphere or PLGA:NMP gel sustained-release formations of rhVEGF$_{165}$ showed an increase in vascular density, diameter and permeability compared to the control-treated animals. In general, these vascular casts showed vessel dilatation and a vascular organization strikingly dissimilar from the hierarchical branching pattern of vessels observed in specimens obtained from control-treated and untreated animals. Marker beads were observed entrapped in the complex vascular plexus at locations that were consistent with the original site of injection. The size of the mouse hind limb was sufficiently small to permit viewing the entire limb in the scanning electron microscope. Although it is possible that marker beads could have migrated from the site of depot injection over the month-long time course of these studies, the marker beads were shown to faithfully define tissue bound and cell-associated fluorescent rhVEGF$_{165}$ for a minimum of 12 hr post IM injection [13]. Additionally, these marker beads served as an internal sizing standard that allowed for an accurate correction of depth of field effects when analyzing scanning electron micrographs.

A single IM injection of rhVEGF$_{165}$-loaded microspheres produces long-term vascular effects

Comparing scanning electron micrographs for time-matched control and rhVEGF$_{165}$-containing microsphere specimens, differences were readily apparent in hind-limb vasculature by three days post-treatment (Fig. 3). Three days after dosing, extensive vessel enlargement and increased vascular permeability were readily observed. Although it is possible that some of the effects observed by IM-injection of the rhVEGF$_{165}$ microsphere formulation could have

been due to a potential inflammatory-foreign body response to the PLGA microspheres themselves [27], this was not the case. A comparison of the vascular casts prepared from hind-limbs treated with placebo microspheres (Fig. 3a) with those of tissues exposed to rhVEGF$_{165}$-loaded microspheres (Fig. 3b) showed a marked enlargement of vessel diameter and tissue effects that were directly associated with the delivery of rhVEGF$_{165}$.

Vessel enlargement was not likely to be due to vessel growth by three days, but rather to dilation of the vessel wall, a loosening of the basement membrane as well as enlarged fenestrations of the capillary wall and increased vascular leakiness or permeability. Indeed, tissue analysis 4 days post-dosing with the rhVEGF$_{165}$ microsphere formulations demonstrated profound swelling in the vicinity of the depot injection site, consistent with an increased vascular leakiness (Fig. 4). Further, immunohistochemical assessment of macrophage recruitment (F4/80 antibody) and endothelial cell activation (up-regulation of CD31 expression) correlated strongly with known actions of rhVEGF$_{165}$ at these depot injection sites (Fig. 4) and their established association with the ability of RHVEGF165 to induce edema [33]. By day 7 post-dosing with rhVEGF$_{165}$-containing PLGA microspheres, standard histological assessment and the distribution of smooth muscle actin (demonstrated by immunohistochemistry) could be used to verify residual edema and the beginnings of nascent vessel maturation at injection sites (Fig. 5). It should be noted that a clinical outcome of hyper-permeable, dilated vasculature is hypotension, the dose limiting endpoint used in dose escalation for the VIVA trial that examined the safety and efficacy of rhVEGF$_{165}$ following intra-coronary artery administration [7; 12].

By day 28 post-dosing, rhVEGF$_{165}$ release from the PLGA microsphere formulation used in these studies should have been completed. Local vasculature of animals treated with

rhVEGF$_{165}$ loaded microspheres, however, was still enlarged (Fig. 6). Further, vascular cast morphology suggested that local vessels were still highly permeable as puddles or filamentous sheets of resin were frequently observed adjacent to these vessels. Although such a finding would be consistent with the hypothesis that these vessels were (more) permeable to the resin that vessels in control preparations where this phenomenon was not observed, it is unclear whether this increased resin leakage was due to weakened vessel walls or the induction of incontinent vasculature. In either case, rhVEGF$_{165}$-microsphere injection sites demonstrated a mixture of vessels with immature morphological characteristics and nascent vasculature maturation at injection sites that could be defined by histochemical and immunohistochemical assessment (e.g. Figs. 4 & 5).

rhVEGF$_{165}$ delivered using PLGA:NMP gel can produce a dose-dependent vascular response

PLGA:NMP gel formulations demonstrated a slower initial *in vivo* release of rhVEGF$_{165}$ than that observed for PLGA microsphere preparations; plasma rhVEGF$_{165}$ levels following PLGA:NMP gel formulation injection peaked at 4 days while the PLGA microsphere formulation produce an initial plasma peak by 6 hr post IM injection. Beyond that point, both gel and microsphere depot formulations appeared to produce similar sustained plasma levels of rhVEGF$_{165}$. These differences in concentration-time profiles did not appear to result in dramatic differences in the observed vasculature outcomes between these two sustained-delivery formulation approaches for the same dose of rhVEGF$_{165}$. This lack of difference in vascular outcomes may have been due to the possibility that the high dose of rhVEGF$_{165}$ being compared (3.6 mg/kg) may have resulted in an overwhelming local effect. Comparison of vascular casts obtained over a 70 day period post-dosing of one of the three rhVEGF$_{165}$ doses

tested using PLGA:NMP gel formulations, however, did demonstrate dose-dependent vascular responses with respect to initial edema, vascular diameter, and vessel leakiness (data not shown).

Low dose rhVEGF$_{165}$ (0.36 mg/kg) PLGA:NMP gel formulation injected IM produced extensive vascular effects by day 7 (Fig. 7a). Some vessels were dilated and most demonstrated extensive tortuosity (Fig. 7a & 7b), a characteristic of nascent vasculature [34]. Vessel architecture at injection sites 7 days post-dosing with 0.72 mg/kg rhVEGF$_{165}$ in PLGA:NMP gel showed vessel dilation similar to low-dose treated tissues (Fig. 8). At day 7, IM injection sites receiving the high dose (3.6 mg/mg) PLGA:NMP gel formulation showed swollen, tortuous vasculature (Fig. 9a) with club-like endings for some vessels (Fig. 9b) consistent with neovascularization [34]. At day 29, vascular casts obtained from mice receiving the lowest dose (0.36 mg/kg) of rhVEGF$_{165}$ in PLGA:NMP gel continued to demonstrate vascular changes consistent with activation of angiogenesis (Fig. 10). Similar responses were observed for the mid (0.72 mg/kg) and high (3.6 mg/kg) doses of rhVEGF$_{165}$ delivered in PLGA:NMP gel formulations at day 29 post-IM injection (data not shown). Importantly, by day 70, vascular tortuosity was strongly correlated with rhVEGF$_{165}$ dose (Fig. 11). Although this dose-dependent effect of rhVEGF$_{165}$ in tortuosity was striking evident at day 70, this vascular parameter was not as evident at earlier times possibly due to the complicating actions of vessel dilation.

DISCUSSION

One major finding of this study is that both rhVEGF$_{165}$-containing PLGA microspheres and PLGA:NMP gel sustained-release dosage forms examined produced local vascular changes anticipated for this potent angiogenic factor. Indices of local edema, vessel swelling and increased permeability correlated with increasing dose and temporal resolution. The sustained rhVEGF$_{165}$ delivery systems described in this study resulted in increased vessel tortuosity that was maintained as long as 70 post-dosing. This is particularly striking as this timeframe suggests that these morphological effects were observed more than one month after the PLGA microspheres or PLGA:NMP gel formulations would likely have ceased to deliver rhVEGF$_{165}$. Further, similar effects were seen in animals in the lowest dose groups, whose rhVEGF$_{165}$ plasma concentrations would likely be below the limit of rhVEGF$_{165}$ ELISA-based detection by two week after doing. These findings provide data that may be useful in the selection of a potential therapeutic drug dose and to determine the optimal duration for an efficacious dosing strategy for the treat of PVD with sustained-release formulations of rhVEGF$_{165}$.

A second important finding of these studies was the demonstration that a polymer-solvent (PLGA:NMP) gel system could be used as a liquid carrier for the sustained release of biologically active rhVEGF$_{165}$ following local injection. Such an approach has several potential advantages over previous efforts to deliver VEGF using solid polymer microspheres or matrix systems. In the present studies we used PLGA microspheres as a system to compare to PLGA:NMP gel delivery systems; a number of other solid systems have been described for the delivery of VEGF. For example, implanted porous PLGA sponges (3x3x4 mm^3) pre-soaked with 2.5-5 μg VEGF and lyophilized have been shown to produce new blood vessels after 2-3 weeks [20]. Similarly, an 85:15 PLGA porous, mineralized scaffold was shown to release

biologically-active rhVEGF$_{165}$ for up to 12 days [35] and facilitate neovascularization [36]. Combining PLGA microspheres with alginate hydrogels containing VEGF has also been found to show promising pre-clinical results for the treatment of PVD following local injection [24]. While these approaches successfully achieve the desired outcome of neovascularization, the PLGA:NMP system offers several advantages: flexibility of dose control through simple changes in drug/carrier ratio, admixing with no additional preparation steps, and simple injection compared to surgical implantation. While PLGA microspheres could have issues with needle-clogging due to particle settling, PLGA nanoparticles loaded with VEGF may reduce this issue, although these smaller particle exhibit a much faster payload release [22] compared to VEGF released from PLGA microspheres [23].

Studies to define *in vitro* release characteristic of rhVEGF$_{165}$ from these sustained release depot formations were performed under biologically-relevant pH and temperature conditions, in order to better simulate what may occur *in vivo*. Cumulative *in vitro* rhVEGF$_{165}$ release from PLGA microspheres was consistent with and somewhat predictive of *in vivo* plasma profiles obtained in rats following IM injection. This was not the case, however, for studies using the PLGA: NMP gel formulation. While this system worked well as a sustained-delivery depot for rhVEGF$_{165}$ *in vivo,* there was very little detectable rhVEGF$_{165}$ release *in vitro,* even though the amount of rhVEGF$_{165}$ to be released was well within assay limits for the drug loading levels anticipated for these release studies. Although not confirmed experimentally, it is thought that the *in vitro* cumulative release assay used for these studies did not contain critical factors/parameters present *in vivo* for the gel system to function properly as a releasing vehicle for rhVEGF$_{165}$. It is likely that the hydrophobic NMP solvent used to prepare the PLGA gel could not efficiently diffuse from the gel *in vitro* due to the extremely low

aqueous solubility of this solvent and the lack of serum proteins containing hydrophobic binding pockets. In the presence of serum/tissue factors that could absorb this hydrophobic solvent it is presumed that NMP would be cleared efficiently from the depot site to allow the gel matrix to set up as a platform for rhVEGF$_{165}$ release *in vivo*.

Plasma concentration-time profiles of rhVEGF$_{165}$ provide only one aspect of the complicated, multi-compartment pharmacokinetic parameters of this potent angiogenic factor. rhVEGF$_{165}$ released from a drug-loaded depot into the tissue surrounding the injection site can bind to extracellular matrix elements through its heparin-binding domain [12; 13]. As rhVEGF$_{165}$ interacts with cell-surface receptors at the injection site, the protein can be internalized by these cells. Since rhVEGF$_{165}$ also acts as a chemotactic molecule, the high local rhVEGF$_{165}$ concentration gradient draws cells that express VEGF receptors on their surface (such as monocytes) to the injection site [13] which further increases the potential number of local rhVEGF$_{165}$ binding sites over time. Such associations could act to sequester low levels of rhVEGF$_{165}$ at the injection site until local concentrations are sufficiently high to overwhelm this local binding compartment and spill over into the systemic (blood) compartment, resulting in a complex non-linear systemic concentration-time profile [12]. Once in the systemic circulation, rhVEGF$_{165}$ would be sequestered by platelets in a manner that further acts to regulate its actions [37].

Despite all the drawbacks and caveats of relying upon plasma concentration-time profile assessments for rhVEGF$_{165}$, this information can be used to compare some performance parameters for sustained delivery formulations. Indeed, that the high dose (3.6 mg/kg) PLGA: NMP gel and PLGA microsphere preparations produced comparable plasma levels of biologically-active (as defined by the KDR receptor-binding ELISA) rhVEGF$_{165}$ and also

produced similar phamacodynamic responses at IM sites supports the possible use of plasma concentrations as a useful assessment tool. A more valuable pharmacokinetic assessment for a sustained rhVEGF$_{165}$ delivery, however, would be to monitor biologically-active rhVEGF$_{165}$ at the depot site for the time course of the study. Indeed, persistent local levels of rhVEGF$_{165}$ that are too low to be measured in the systemic circulation by the KDR binding ELISA could be sufficient to elicit and sustain a desired angiogenic response. Although some progress has been made toward assessing the biological activity of rhVEGF$_{165}$ at an injection site over a 12 hr time frame [13], it is unlikely that current techniques could be sufficiently sensitive to monitor local rhVEGF$_{165}$ concentrations beyond the initial phase of depot protein release due to the associated complexities of extracellular matrix and receptor binding.

In the absence of pharmacokinetic data at the depot site, pharmacodynamic parameters become extremely valuable for comparing the performance of different sustained delivery formulations of rhVEGF$_{165}$. This is particularly important in light of studies suggesting PVD may be due to reduced responsiveness to neovascularization that can be overcome by local VEGF delivery [38]. In the present studies, we analyzed vascular casts by scanning electron microscopy (SEM) to evaluate the extent and persistence of rhVEGF$_{165}$ actions. This method has probably the highest resolution of any current technique for the pharmacodynamic assessment of vascular response to an angiogenic factor such as rhVEGF$_{165}$. While others have used SEM and transmission electron microscopy to assess the angiogenic potential of VEGF-polymer matrices [20], SEM analysis of vascular casts permits visual observation of a number of vessel parameters such as diameter, permeability, branching, tortuosity, etc [33]. We also found that the use of 30 nm-diameter fluorescent latex beads as a co-injected element to not only assist in the localization of injection sites (even weeks after administration) but also to

provide an internal size standard to allow easy calibration throughout the entire depth of an SEM micrograph. One drawback of vascular casting, however, is that this is a terminal procedure, allowing for the capture of only one time point and does not allow for assessment over time in the same animal. Capillary density changes over time can be examined by videomicroscopy and vasodilation can be assessed by laser Doppler flowmetry [39]. Ultrasound biomicroscopy or micro-ultrasound imaging and micro-computed tomography analysis have also been used to assess temporal changes in functional blood flow [40]. Thus, there are vital methods that can be used to correlate with terminal microscopy methods such as vascular casts analyzed by SEM.

Although functional blood flow parameters were not correlated with the vascular casting data obtained in the current study, several other parameters associated with vascular function and angiogenesis were evaluated. Immunohistochemistry using antibodies to VEGF and CD34 have been used to correlate microvessel density with non-invasive measurements of vascular function [41]. We correlated vascular cast information with vessel density using CD31 (PECAM1) and smooth muscle actin expression, as well as F4/80 expression to monitor monocyte/macrophage infiltration, and hematoxylin/eosin staining to assess local edema. Although all of these methods are prone to some level of artifact and uncertainty, co-injected 30 nm-diameter fluorescent latex beads were used to verify depot injection sites and ensure that correct tissue sites were analyzed by these various techniques. Overall, the data presented in this report supports the contention that a sustained, local delivery of rhVEGF$_{165}$ can be achieved from a single injection using formulations of either PLGA microspheres or a gel composed of PLGA/NMP. While both of these approaches provide an method to incite neovascularization using sustained delivery systems that can be injected rather than surgically implanted, flexibility

of the PLGA:NMP system may be particularly useful for dose-ranging studies in clinical settings. Obviously, further evaluation in relevant pre-clinical models would be important to support the potential of such formulations for the clinical treatment of PVD.

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REFERENCES

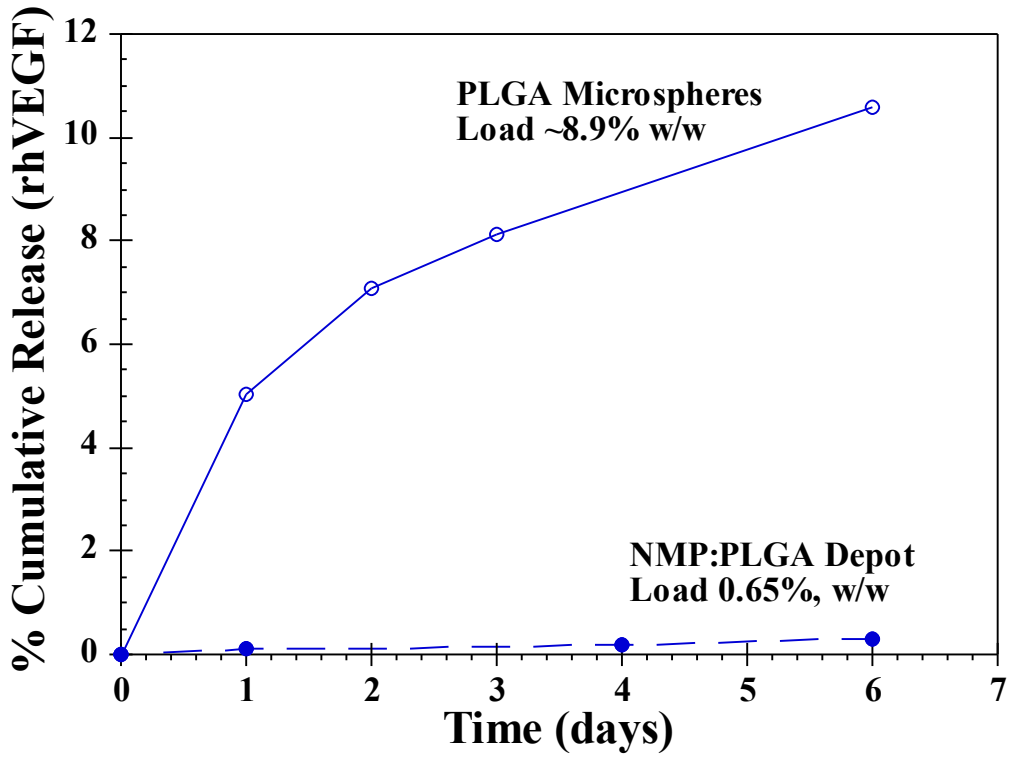
1. N. Ferrara, Vascular endothelial growth factor and the regulation of angiogenesis. Recent Prog Horm Res 55 (2000) 15-35.
2. L. Chen, A. Endler, and F. Shibasaki, Hypoxia and angiogenesis: regulation of hypoxia-inducible factors via novel binding factors. Exp Mol Med 41 (2009) 849-57.
3. P. Carmeliet, Mechanisms of angiogenesis and arteriogenesis. Nat Med 6 (2000) 389-95.
4. J. Folkman, Angiogenesis: an organizing principle for drug discovery? Nat Rev Drug Discov 6 (2007) 273-86.
5. A.M. Byrne, D.J. Bouchier-Hayes, and J.H. Harmey, Angiogenic and cell survival functions of vascular endothelial growth factor (VEGF). J Cell Mol Med 9 (2005) 777-94.
6. C. Bauters, T. Asahara, L.P. Zheng, S. Takeshita, S. Bunting, N. Ferrara, J.F. Symes, and J.M. Isner, Site-specific therapeutic angiogenesis after systemic administration of vascular endothelial growth factor. J Vasc Surg 21 (1995) 314-24; discussion 324-5.
7. T.D. Henry, K. Rocha-Singh, J.M. Isner, D.J. Kereiakes, F.J. Giordano, M. Simons, D.W. Losordo, R.C. Hendel, R.O. Bonow, S.M. Eppler, T.F. Zioncheck, E.B. Holmgren, and E.R. McCluskey, Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease. Am Heart J 142 (2001) 872-80.
8. C. Bauters, T. Asahara, L.P. Zheng, S. Takeshita, S. Bunting, N. Ferrara, J.F. Symes, and J.M. Isner, Physiological assessment of augmented vascularity induced by VEGF in ischemic rabbit hindlimb. Am J Physiol 267 (1994) H1263-71.
9. F.W. Sellke, M. Tofukuji, R.J. Laham, J. Li, M.D. Hariawala, S. Bunting, and M. Simons, Comparison of VEGF delivery techniques on collateral-dependent microvascular reactivity. Microvasc Res 55 (1998) 175-8.
10. S.P. Hopkins, J.P. Bulgrin, R.L. Sims, B. Bowman, D.L. Donovan, and S.P. Schmidt, Controlled delivery of vascular endothelial growth factor promotes neovascularization and maintains limb function in a rabbit model of ischemia. J Vasc Surg 27 (1998) 886-94.
11. J.W. Ji, F. Mac Gabhann, and A.S. Popel, Skeletal muscle VEGF gradients in peripheral arterial disease: simulations of rest and exercise. Am J Physiol Heart Circ Physiol 293 (2007) H3740-9.
12. S.M. Eppler, D.L. Combs, T.D. Henry, J.J. Lopez, S.G. Ellis, J.H. Yi, B.H. Annex, E.R. McCluskey, and T.F. Zioncheck, A target-mediated model to describe the pharmacokinetics and hemodynamic effects of recombinant human vascular endothelial growth factor in humans. Clin Pharmacol Ther 72 (2002) 20-32.
13. A.L. Daugherty, and R.J. Mrsny, Local tissue distribution and cellular fate of vascular endothelial growth factor (VEGF) following intramuscular injection. J Drug Target 18 (2010) 27-35.

14. S. Yla-Herttuala, T.T. Rissanen, I. Vajanto, and J. Hartikainen, Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. J Am Coll Cardiol 49 (2007) 1015-26.
15. J. Folkman, Angiogenesis. Annu Rev Med 57 (2006) 1-18.
16. T.P. Richardson, M.C. Peters, A.B. Ennett, and D.J. Mooney, Polymeric system for dual growth factor delivery. Nat Biotechnol 19 (2001) 1029-34.
17. P. Losi, E. Briganti, A. Magera, D. Spiller, C. Ristori, B. Battolla, M. Balderi, S. Kull, A. Balbarini, R. Di Stefano, and G. Soldani, Tissue response to poly(ether)urethane-polydimethylsiloxane-fibrin composite scaffolds for controlled delivery of pro-angiogenic growth factors. Biomaterials 31 (2010) 5336-44.
18. Q. He, Y. Zhao, B. Chen, Z. Xiao, J. Zhang, L. Chen, W. Chen, F. Deng, and J. Dai, Improved cellularization and angiogenesis using collagen scaffolds chemically conjugated with vascular endothelial growth factor. Acta Biomater 7 (2011) 1084-93.
19. Q. Sun, R.R. Chen, Y. Shen, D.J. Mooney, S. Rajagopalan, and P.M. Grossman, Sustained vascular endothelial growth factor delivery enhances angiogenesis and perfusion in ischemic hind limb. Pharm Res 22 (2005) 1110-6.
20. A.E. Elcin, and Y.M. Elcin, Localized angiogenesis induced by human vascular endothelial growth factor-activated PLGA sponge. Tissue Eng 12 (2006) 959-68.
21. F.R. Formiga, B. Pelacho, E. Garbayo, G. Abizanda, J.J. Gavira, T. Simon-Yarza, M. Mazo, E. Tamayo, C. Jauquicoa, C. Ortiz-de-Solorzano, F. Prosper, and M.J. Blanco-Prieto, Sustained release of VEGF through PLGA microparticles improves vasculogenesis and tissue remodeling in an acute myocardial ischemia-reperfusion model. J Control Release 147 (2010) 30-7.
22. J.S. Golub, Y.T. Kim, C.L. Duvall, R.V. Bellamkonda, D. Gupta, A.S. Lin, D. Weiss, W. Robert Taylor, and R.E. Guldberg, Sustained VEGF delivery via PLGA nanoparticles promotes vascular growth. Am J Physiol Heart Circ Physiol 298 (2010) H1959-65.
23. O. Karal-Yilmaz, M. Serhatli, K. Baysal, and B.M. Baysal, Preparation and in vitro characterization of vascular endothelial growth factor (VEGF)-loaded poly(D,L-lactic-co-glycolic acid) microspheres using a double emulsion/solvent evaporation technique. J Microencapsul 28 (2011) 46-54.
24. J. Lee, S.H. Bhang, H. Park, B.S. Kim, and K.Y. Lee, Active blood vessel formation in the ischemic hindlimb mouse model using a microsphere/hydrogel combination system. Pharm Res 27 (2010) 767-74.
25. J.L. Cleland, E.T. Duenas, A. Park, A. Daugherty, J. Kahn, J. Kowalski, and A. Cuthbertson, Development of poly-(D,L-lactide-co-glycolide) microsphere formulations containing recombinant human vascular endothelial growth factor to promote local angiogenesis. J Control Release 72 (2001) 13-24.
26. J.M. Anderson, A. Rodriguez, and D.T. Chang, Foreign body reaction to biomaterials. Semin Immunol 20 (2008) 86-100.
27. A.L. Daugherty, J.L. Cleland, E.M. Duenas, and R.J. Mrsny, Pharmacological modulation of the tissue response to implanted polylactic-co-glycolic acid mocrospheres. Eur J Pharm Biopharm 44 (1997) 89-102.

28. W.K. Ward, A Review of the Foreign-body Response to Subcutaneously-implanted Devices: The Role of Macrophages and Cytokines in Biofouling and Fibrosis. Journal of Diabetes Science and Technology 2 (2008) 768-777.
29. G.C. Douglas, and F.E. Hossler, Vascular anatomy of the rabbit ureter. Anat Rec 242 (1995) 47-56.
30. F. Steinberg, M.A. Konerding, and C. Streffer, The vascular architecture of human xenotransplanted tumors: histological, morphometrical, and ultrastructural studies. J Cancer Res Clin Oncol 116 (1990) 517-24.
31. T. Kofidis, D. Nolte, A.R. Simon, A. Metzakis, L. Balsam, R. Robbins, and A. Haverich, Restoration of blood flow and evaluation of corresponding angiogenic events by scanning electron microscopy after a single dose of VEGF in a model of peripheral vascular disease. Angiogenesis 5 (2002) 87-92.
32. H.B. Ravivarapu, K. Burton, and P.P. DeLuca, Polymer and microsphere blending to alter the release of a peptide from PLGA microspheres. Eur J Pharm Biopharm 50 (2000) 263-70.
33. D.M. McDonald, and P.L. Choyke, Imaging of angiogenesis: from microscope to clinic. Nat Med 9 (2003) 713-25.
34. M. Neeman, Preclinical MRI experience in imaging angiogenesis. Cancer Metastasis Rev 19 (2000) 39-43.
35. W.L. Murphy, M.C. Peters, D.H. Kohn, and D.J. Mooney, Sustained release of vascular endothelial growth factor from mineralized poly(lactide-co-glycolide) scaffolds for tissue engineering. Biomaterials 21 (2000) 2521-7.
36. W.L. Murphy, C.A. Simmons, D. Kaigler, and D.J. Mooney, Bone regeneration via a mineral substrate and induced angiogenesis. J Dent Res 83 (2004) 204-10.
37. J. Folkman, T. Browder, and J. Palmblad, Angiogenesis research: guidelines for translation to clinical application. Thromb Haemost 86 (2001) 23-33.
38. G. Cobellis, C. Maione, C. Botti, A. Coppola, A. Silvestroni, S. Lillo, V. Schiavone, A.M. Molinari, and V. Sica, Beneficial effects of VEGF secreted from stromal cells in supporting endothelial cell functions: therapeutic implications for critical limb ischemia. Cell Transplant. 19 (2010) 1425-37.
39. R.T. de Jongh, E.H. Serne, I.J. RG, and C.D. Stehouwer, Microvascular function: a potential link between salt sensitivity, insulin resistance and hypertension. J Hypertens 25 (2007) 1887-93.
40. A.M. Cheung, A.S. Brown, V. Cucevic, M. Roy, A. Needles, V. Yang, D.J. Hicklin, R.S. Kerbel, and F.S. Foster, Detecting vascular changes in tumour xenografts using micro-ultrasound and micro-ct following treatment with VEGFR-2 blocking antibodies. Ultrasound Med Biol 33 (2007) 1259-68.
41. F. Graziani, M. Vano, P. Viacava, A. Itro, G. Tartaro, and M. Gabriele, Microvessel density and vascular endothelial growth factor (VEGF) expression in human radicular cysts. Am J Dent 19 (2006) 11-4.

FIGURES

Figure 1. Percent cumulative *in vitro* release rates of rhVEGF from PLGA microspheres (open circles) and PLGA: NMP gel (solid circles). Microspheres had 8.9% w/w loading of rhVEGF and the PLGA: NMP gel had 0.65% w/w loading of rhVEGF.



Release buffer: 10 mM Histidine, 140 mM NaCl, 0.02% P20, 0.02% NaN_3, pH 7.0
Protein Form. in µspheres: 10 mg/mL VEGF, 1 mg/mL trehalose, 0.01% P20, 5mM Succinate, pH 5.0
Protein Form. in NMP:PLGA: 10 mg/mL VEGF, 10 mg/mL trehalose, 0.03% P80, 10 mM Histidine, pH 6.0

Figure 2. Pharmacokinetic profiles of plasma rhVEGF concentrations over time of normal rats following intramuscular dosing with 3.6 mg/kg rhVEGF loaded PLGA microspheres (circles) and 3.6 mg/kg rhVEGF in PLGA:NMP gel (triangles).

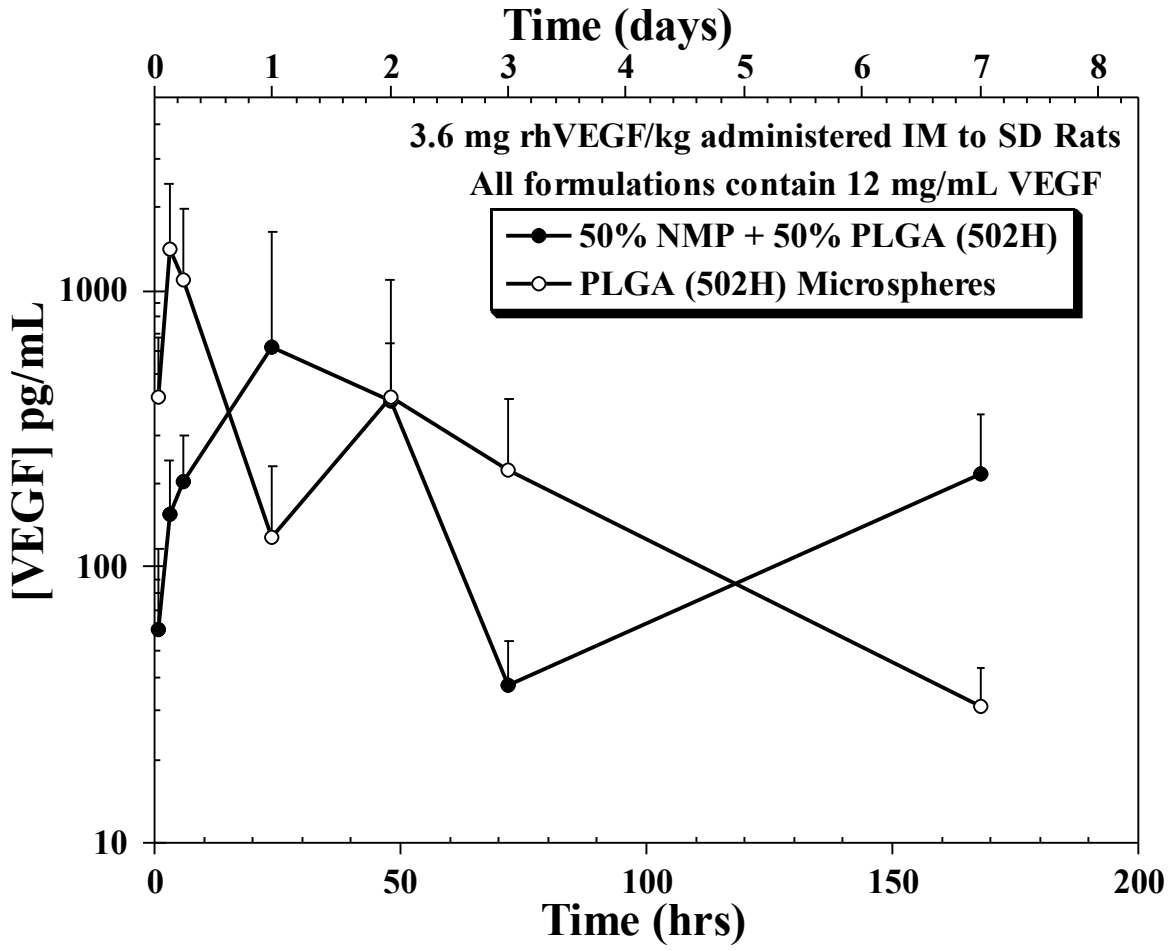


Figure 3. Vascular casts of intramuscular injection sites three days after dosing with control microspheres (a) or 3.6 mg/kg rhVEGF loaded-microsphere (b). Note vessel dilation in response to rhVEGF exposure. Co-injected polystyrene divinylbenzene (non-dissolving) beads (30 μm) are denoted by arrows.

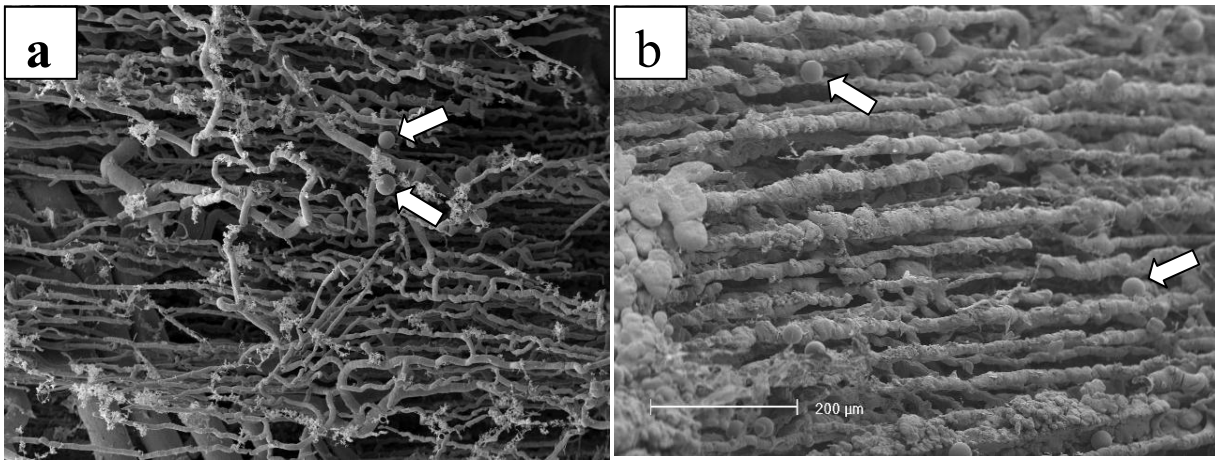


Figure 4. Distribution of macrophage infiltration and endothelial cell activation as a result of local, sustained delivery of rhVEGF. Serial tissue sections obtained from an intramuscular injection site of 3.6 mg/kg rhVEGF in PLGA microspheres four days post-dose were labeled with (a) the F4/80 antibody specific for mouse macrophages or (b) the cell surface marker CD31 associated with endothelial cell proliferation. The injection site is indicated by an arrow.

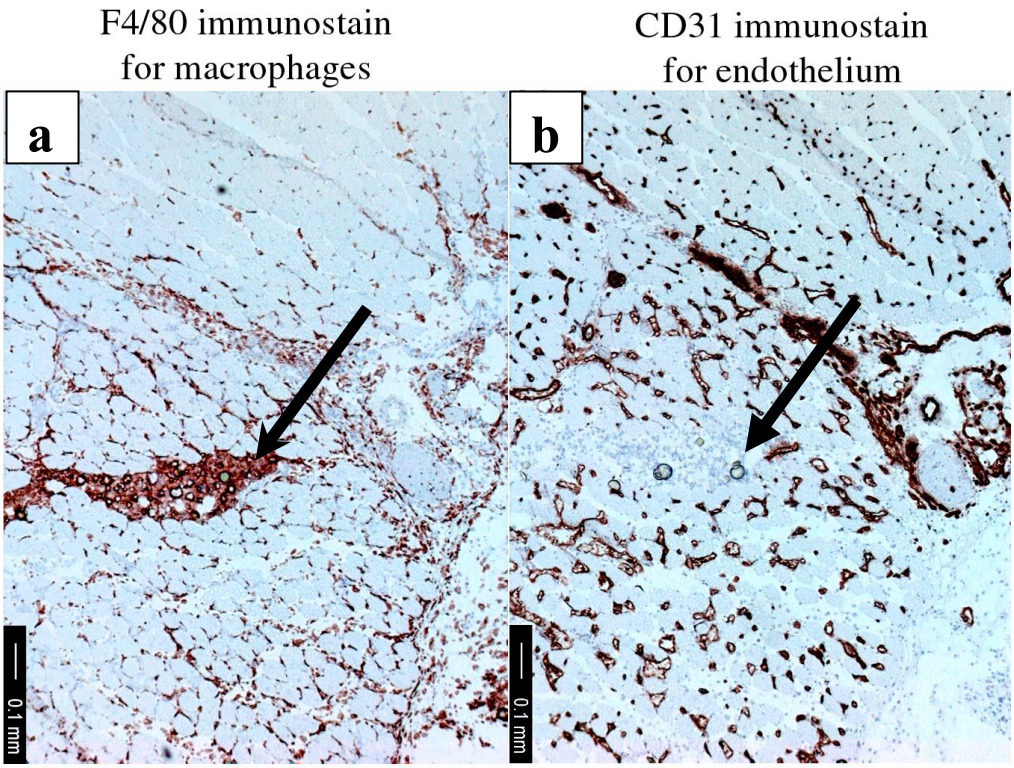


Figure 5. Induction of local edema and nascent vasculature following local, sustained delivery of rhVEGF. Tissues sections obtained from an intramuscular injection site of 3.6 mg/kg rhVEGF in PLGA microspheres seven days post-dose were (a) stained with hematoxylin and eosin to assess local edema and cellular infiltration or (b) labeled with an antibody specific for mouse smooth muscle actin to delineate the presence of nascent vasculature.

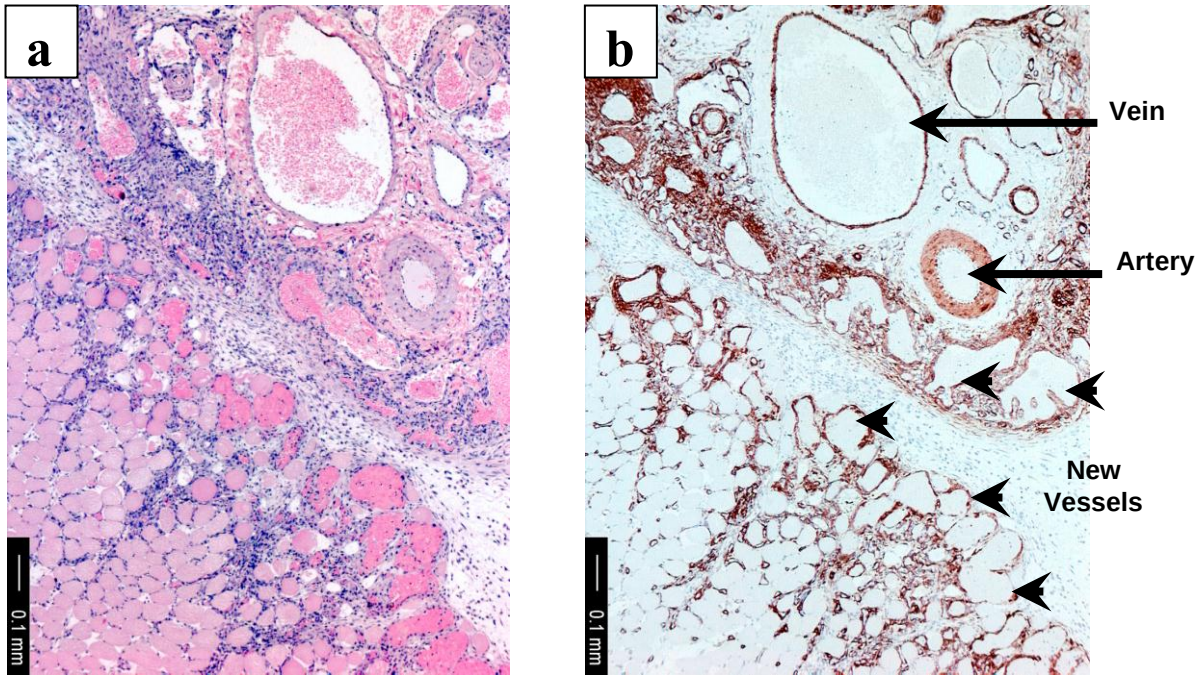


Figure 6. Vascular casts of intramuscular injection site 28 days after dosing IM with 3.6 mg/kg rhVEGF loaded microspheres. Note increased leakage of injected resin. Co-injected polystyrene divinylbenzene beads (30 μm) are denoted by arrows.

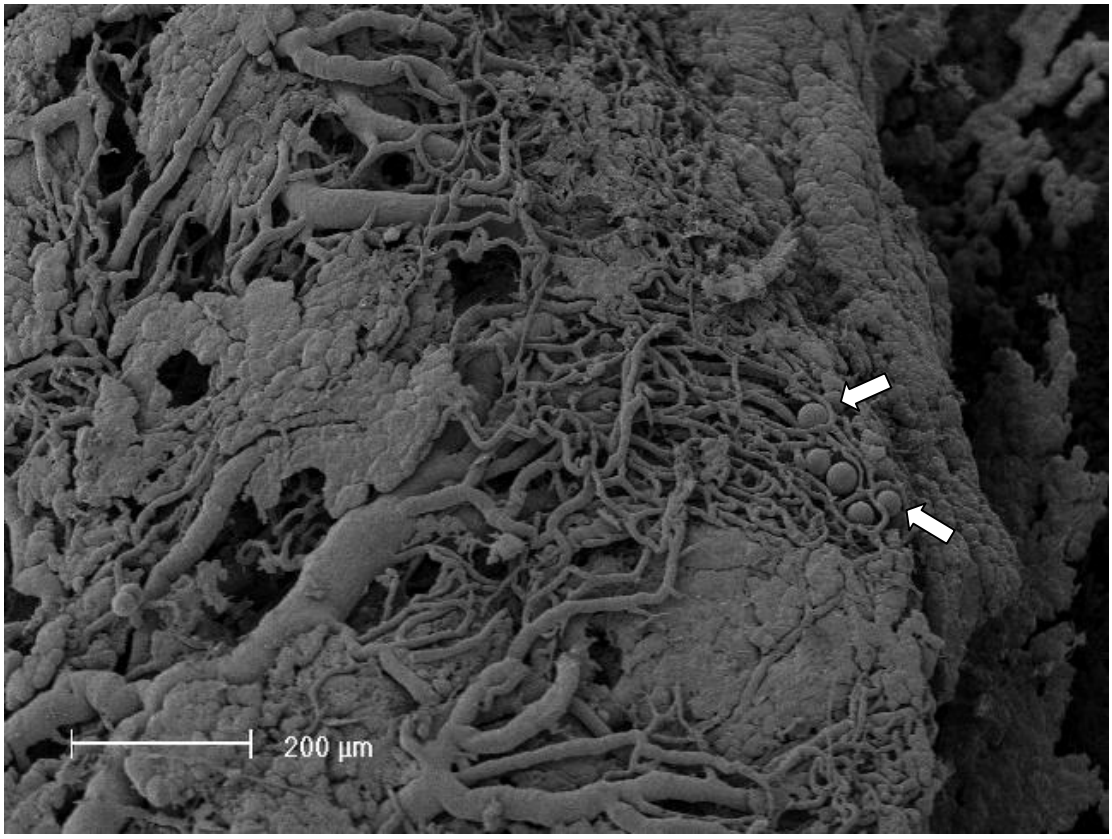


Figure 7. Vascular casts of intramuscular injection site 7 days after dosing with 0.36 mg/kg rhVEGF in PLGA:NMP gel shown at (a) lower and (b) higher magnifications highlighting vessel tortuosity. Co-injected polystyrene divinylbenzene beads (30 μm) are denoted by arrows.

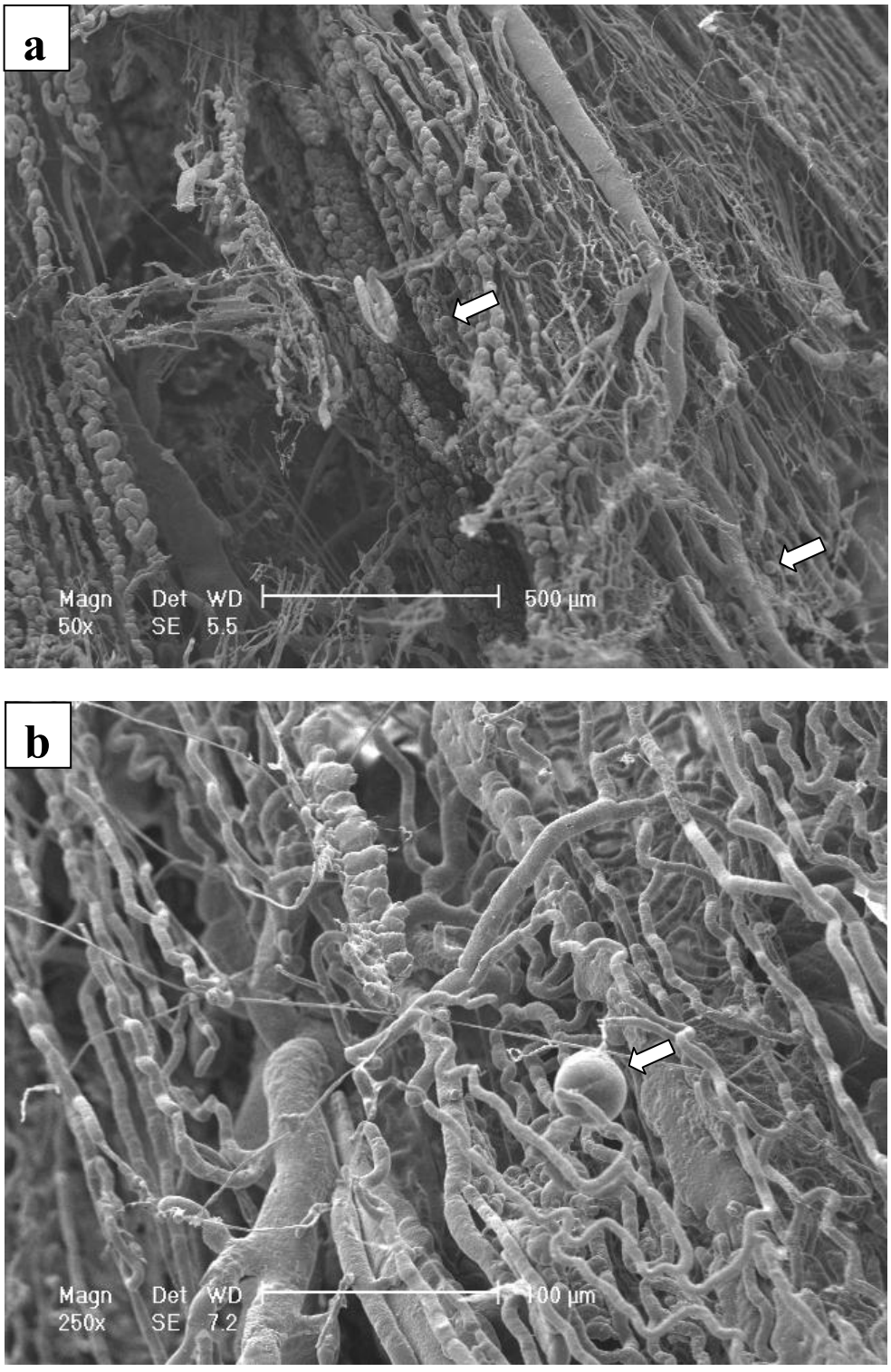


Figure 8. Vascular casts of intramuscular injection site 7 days after dosing with 0.72 mg/kg rhVEGF in PLGA:NMP gel. Co-injected polystyrene divinylbenzene beads (30 μm) are denoted by arrows.

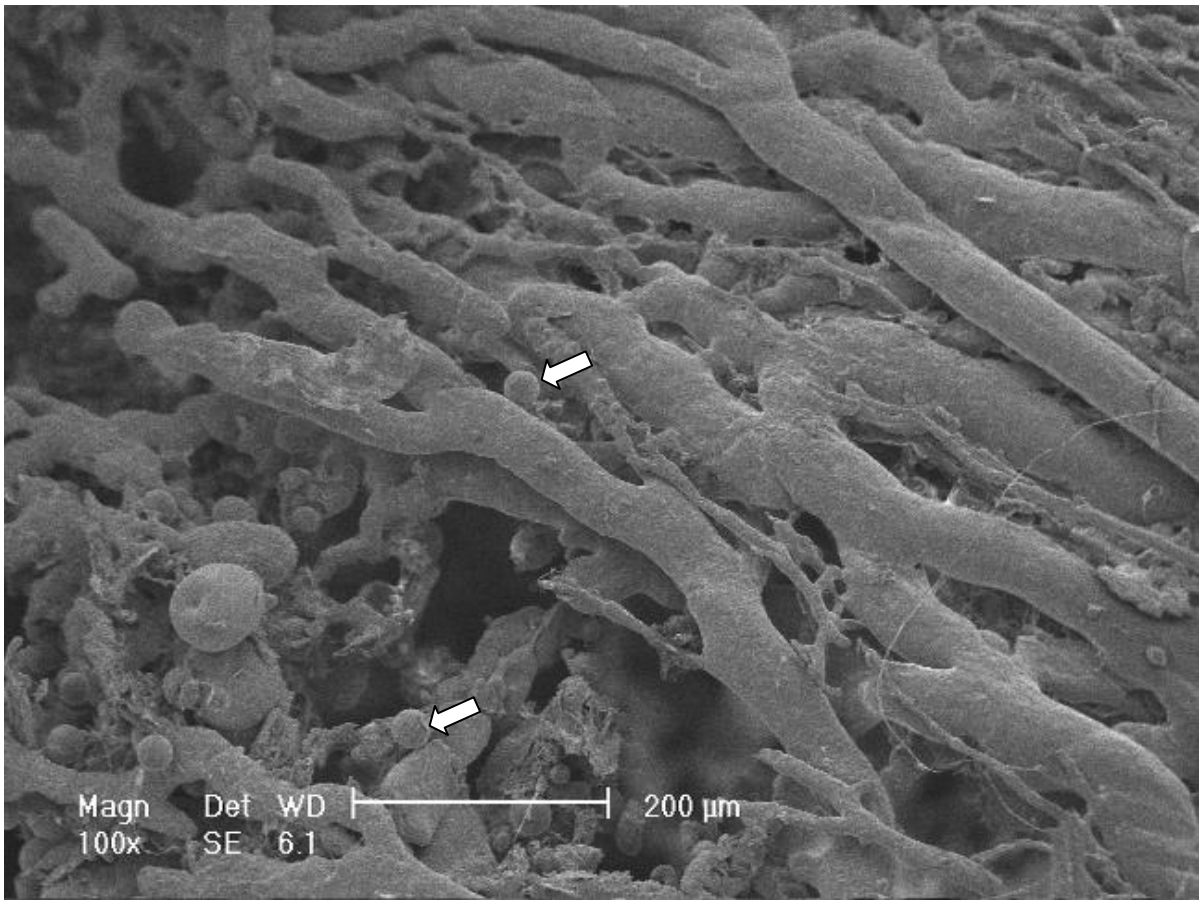
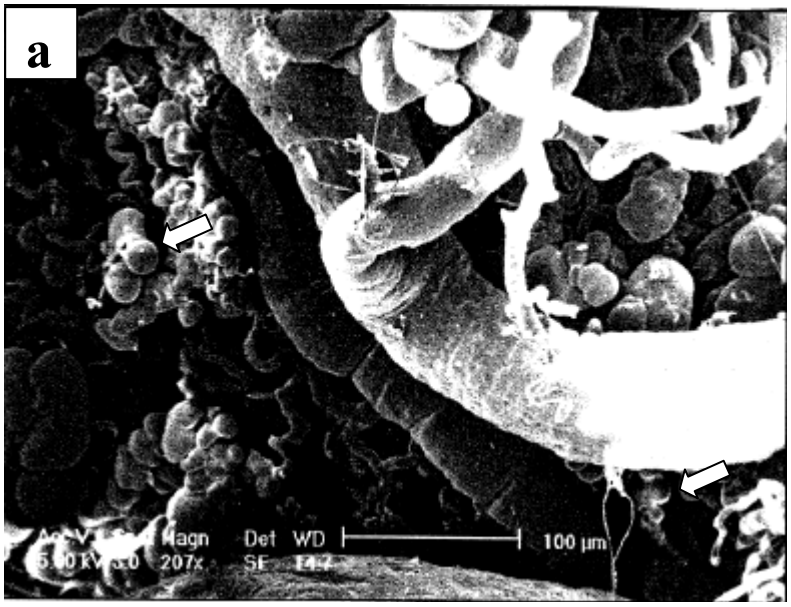


Figure 9. Vascular casts of intramuscular injection site 7 days after dosing with 3.6 mg/kg rhVEGF in PLGA:NMP gel. Examples of (a) vascular leakiness and vessel dilation as well as (b) club-like vessel endings showing individual endothelial cell organization at sites of nascent vessel formation are shown. Note Co-injected polystyrene divinylbenzene beads (30 μm) are denoted by arrows.



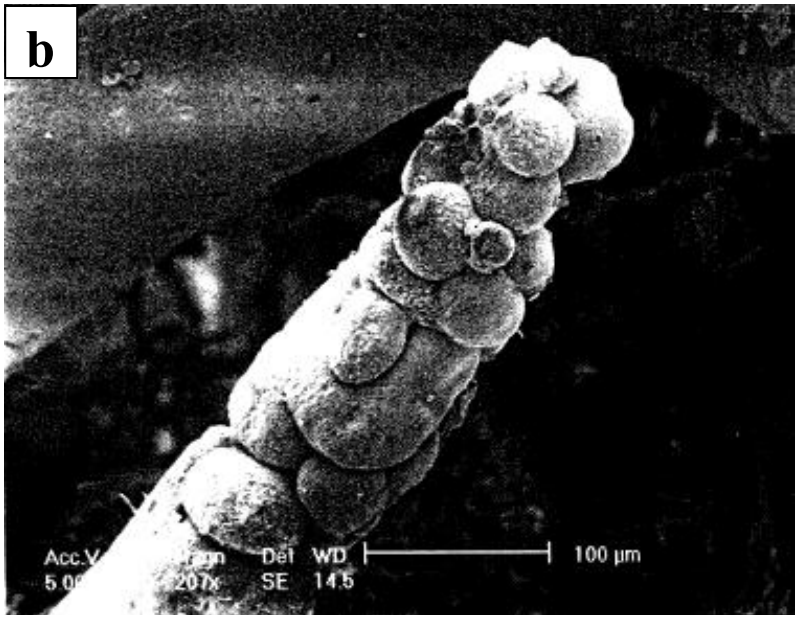


Figure 10. Vascular casts of intramuscular injection site 29 days after dosing with 0.36 mg/kg rhVEGF in PLGA:NMP gel. Co-injected polystyrene divinylbenzene beads (30 μm) are denoted by arrows.

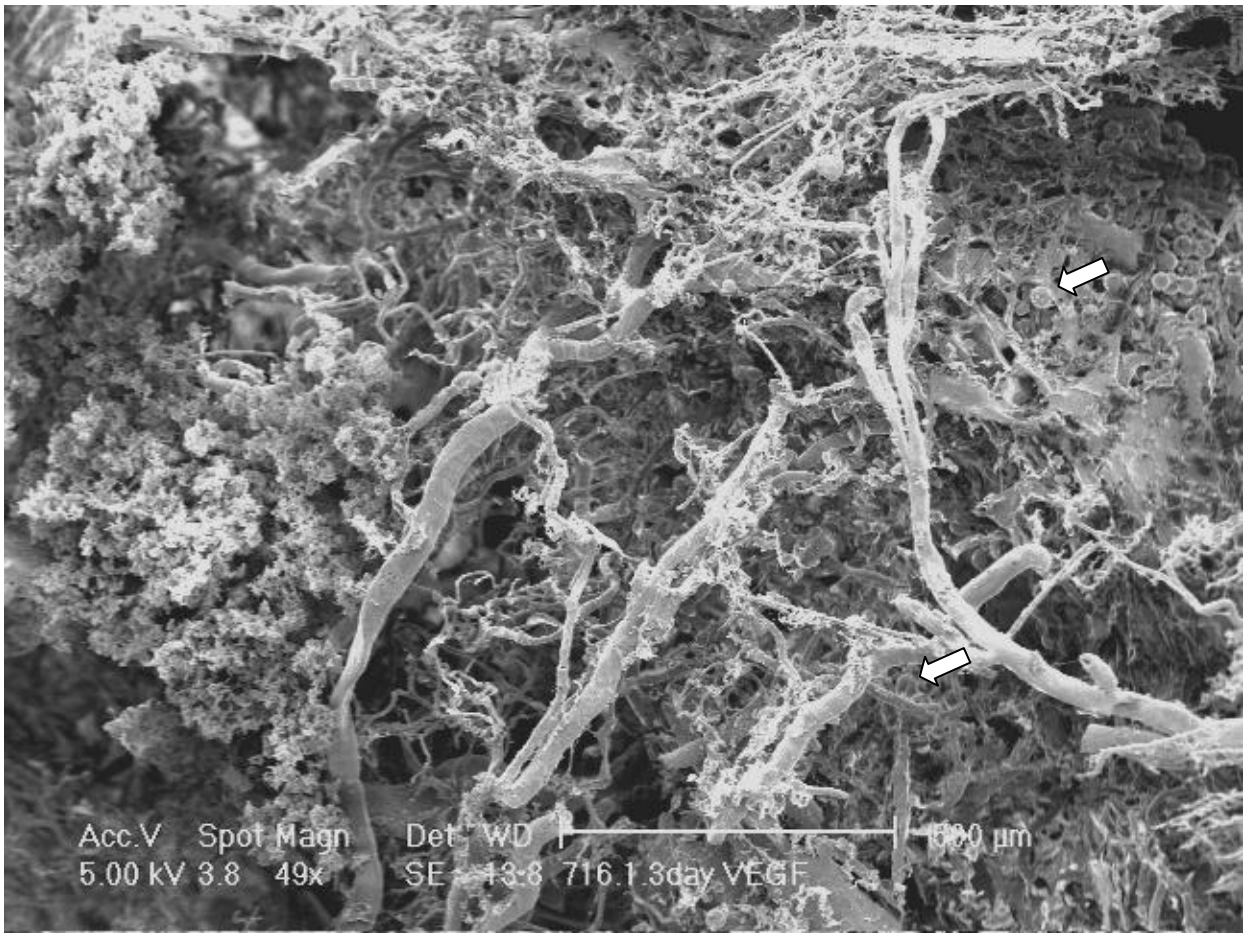


Figure 11. Vascular casts of intramuscular injection site 70 days after dosing with 3.6 mg/kg rhVEGF in PLGA:NMP gel showing examples of the tortuous, but organized, nature of the vascular bed at (a) lower and (b) higher magnifications.