Temporally regulated delivery of VEGF *in vitro* and *in vivo*

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Abstract: The exposure duration and tissue distribution will likely dictate the success of vascular endothelial growth factor (VEGF) in therapeutic angiogenesis. We hypothesized that these variables can be regulated via the manner in which the VEGF is incorporated into polymer constructs (formed with a gas foaming technique) used for its delivery. VEGF was incorporated directly into poly(lactide-*co*-glycolide) (PLG) scaffolds or pre-encapsulated in PLG microspheres used to fabricate scaffolds. Protein release kinetics and tissue distribution were determined using iodinated VEGF. VEGF was positioned predominantly adjacent to scaffold pores when incorporated directly and was released rapidly (40–60% in 5 days). Pre-encapsulation led to the VEGF being more deeply embedded and resulted in a delayed release. Alterations in polymer composition, scaffold size, and matrix composition generated minor variations in release kinetics. *In vivo*, the released VEGF generated local protein concentrations above 10 ng/mL at distances up to 2 cm from the implant site for the 21 days of the experiment, with negligible release into the systemic circulation, and significantly enhanced local angiogenesis. These data indicate that VEGF can be administered in a sustained and localized fashion *in vivo*, and the timing of VEGF delivery can be altered with the mechanism of incorporation into polymer scaffolds used for its delivery. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 79A: 176–184, 2006

Key words: drug delivery; poly(lactide-*co*-glycolide) (PLG); angiogenesis; localized delivery; protein distribution

INTRODUCTION

Coronary heart disease (CHD) is the leading single cause of death among Americans, and significant attention has turned toward the development of new therapies for the millions of patients diagnosed with this disease. Standard treatment of CHD often includes lifestyle changes (i.e., weight and stress reduction, nutrition, exercise, eliminate smoking) and various medications. Patients suffering with more severe cases require the invasive mechanical procedures of angioplasty or bypass surgery to restore perfusion in the diseased areas. However, many patients are not viable candidates for these procedures because of age and the presence of other diseases (i.e., diabetes, obesity, and hypertension). For those who undergo these surgical treatments, a high percentage suffer from restenosis while for others revascularization is insufficient.[1] New therapies may eventually allow one to conquer this disease. Therapeutic angiogenesis, the promotion of neovascularization to restore adequate perfusion in ischemic regions, offers enormous promise for individuals suffering from CHD.

Angiogenesis is the development of nascent blood vessels via sprouting from the sides and ends of preexisting vessels or by intussusception, longitudinal division of existing vessels with periendothelial cells.[2] This normal, physiologic process occurs during events such as embryonic development, wound healing, inflammation, and female reproduction. The major stages of angiogenesis entail the activation, migration, and proliferation of endothelial cells (EC) (the cells that form the lining of blood vessels), the assembly of these cells into tube-like structures forming immature vessels, the stabilization of these vessels through EC association with mural cells (pericytes and smooth muscle cells), and deposition of extracellular matrix around the maturing blood vessels.[3,4]

Angiogenesis is a complex, multistage cascade of events involving numerous proteases, cytokines, cell

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types, and growth factors, all functioning in a concerted manner. One of the most widely studied regulators of this process is vascular endothelial growth factor (VEGF).[5] This 46-kDa dimeric glycoprotein is a known mitogen of ECs. VEGF initiates EC migration and proliferation during angiogenesis, causing immature tube-like structures to branch from mature blood vessels. If this signal is removed, these nascent vessels regress. Additionally, VEGF, once introduced into a host environment, has a half-life of 90 min.[6] In order to provide tissues with adequate exposure to VEGF to elicit a cellular response, a constant supply of the soluble signal is most likely required. However, owing to the potent mitogenic nature of VEGF, systemic exposure may promote undesired vascularization in nontarget sites as well as enhance pathological conditions (i.e., tumor growth, retinopathies) in distant areas. Thus, for therapeutic purposes, the presence of VEGF must not only be sustained, but also be localized to the ischemic tissue region.

In this report, we focus on developing a polymeric system capable of controlling the kinetics of VEGF release as well as demonstrating that such an approach can allow for sustained and localized presence of the protein *in vivo*. In previous investigations, a poly(lactide-*co*-glycolide) (PLG) system was developed that allowed a sustained delivery of biologically active VEGF *in vitro* while greatly enhancing neovascularization *in vivo*.[7–9] Based on these studies, we hypothesize that varying the approach of VEGF incorporation into the PLG scaffold will enable us to control how the protein is distributed in the matrix and, thus, how it is released. Furthermore, we investigate whether this strategy provides the capability to release VEGF in a manner that creates a local concentration that could attract blood vessels in surrounding tissue without having significant systemic exposure to the protein.

MATERIALS AND METHODS

PLG was purchased from either Medisorb-Alkermes (Cambridge, MA; 85:15) or Boehringer Ingelheim [Petersburg, VA, 75:25 Resomer RG752 (intrinsic viscosity = 0.24 dL/g, MW = 16 kDa), Resomer RG755 (intrinsic viscosity = 0.59 dL/g, MW = 63 kDa), Resomer RG756 (intrinsic viscosity = 0.80 dL/g, MW = 98 kDa), and 50:50 Resomer RG502 (intrinsic viscosity = 0.20 dL/g)]. Iodinated VEGF (^{125}I VEGF, specific activity = 3500–4600 μCi/mmol) was obtained from Perkin Elmer (Boston, MA). CB17 SCID mice were procured from Taconic Farms Inc. (Germantown, NY) and C57Bl/6J mice from Jackson Laboratories (Bar Harbor, Maine). Bovine serum albumin conjugates, Alexa Fluor 488 and Texas Red, were purchased from Molecular Probes (Eugene, OR) and Collagenase Type II was bought from Worthington Biochemical Corporation (Lakewood, NJ). Other supplies were obtained from Sigma (St. Louis, MO), unless noted otherwise.

Microspheres and scaffold preparation

A variety of PLGs, differing in composition (lactide to glycolide ratio) and molecular weight, were used to prepare microspheres (particle size, 5–50 μm), incorporating growth factor, using a double emulsion (water/oil/water) process as previously described.[10] An aqueous growth factor solution was mixed with PLG dissolved in ethyl acetate to form the first emulsion. A 1% (w/v) solution of poly(vinyl) alcohol (PVA) in ethyl acetate was combined with the first emulsion, vortexed, and added to a stirring solution of 7% (w/v) ethyl acetate, 3% (w/v) PVA, and water. The ethyl acetate in solution evaporated during the 3-h stir period, and the microspheres were collected by filtration and lyophilized.

Scaffolds were prepared from particulate PLG and PLG microspheres using a gas foaming/particulate leaching process, as previously described.[11,12] Scaffolds were fabricated from either a mix of particulate PLG (85:15, ground to an average diameter of 125 μm, ±^{125}I VEGF, 0.2 μCi) and up to 30% of total polymer mass of PLG microspheres (±^{125}I VEGF, 0.2 μCi) or fabricated entirely from microspheres (±^{125}I VEGF, 0.2 μCi). In either case, the polymer in the form of particles, microspheres, or a mixture of both was combined with NaCl particles (diameter, 250–425 μm) and 1% (w/v) alginate solution. The alginate serves to increase protein incorporation and functions as a stabilizer.[7] This mixture was lyophilized and pressed into a pellet using a Carver press. The scaffolds were placed under high pressure CO_2 gas and allowed to equilibrate. The pressure was rapidly returned to ambient conditions leading to a thermodynamic instability and causing the polymer, whether in the form of particulate or microspheres, to foam and create an interconnected structure around the NaCl. Both types of particles foam and fuse together to create the scaffold, and no distinct particles or microspheres are present in the scaffold after this processing. The NaCl was leached in a $CaCl_2$ solution to create a macroporous structure.[13] Scaffolds were 13 mm in diameter and 3 mm thick (40 mg total polymer and 760 mg NaCl) or 4.7 mm in diameter and 1.5 mm thick (3 mg total polymer and 50 mg NaCl).

In vitro protein release

^{125}I VEGF was incorporated into the scaffolds as a tracer, and scaffolds (n = 4–8) were placed in phosphate buffer saline (PBS) containing calcium and magnesium, and incubated at 37°C. At set time points, a sample of the buffer was removed and analyzed using a Packard γ counter. The remaining liquid was removed and discarded, and a fresh aliquot of PBS was added to the scaffold. The quantity of protein released from the scaffold was determined by comparing it to the total quantity of radioactivity initially present in each sample.

Confocal microscopy imaging of protein distribution

Microspheres (encapsulating BSA conjugated fluorophor, Texas Red) were prepared from 75:25 PLG (MW = 63 kDa) and added to a combination of 85:15 PLG particles, the BSA conjugated fluorophor Alexa Fluor 488, and alginate, to fabricate scaffolds using the gas foaming/particulate leaching method described earlier. Scaffolds were analyzed using a BioRad confocal microscope (Hercules, CA) and a dual channel filter. A series of images (*z*-stack), 4–8 μm apart in depth, were taken of several areas of scaffolds, totaling ~100 μm in depth for each area. Images of the scaffold surface, as well as the interior, were examined.

In vivo protein release

Mice (n = 5 per time point) were anesthetized with a mixture of ketamine and xylazine. A small incision was made on the dorsal side of the rodent, and a single scaffold was subcutaneously implanted into the pocket. Prior to implantation, each scaffold was submerged in 100% ethanol for 15 min. This sterilization technique did not significantly influence the bioactivity of the protein incorporated and released from the scaffolds, as previously determined in EC proliferation assays[8,9] with this system. After removing the ethanol, the scaffolds were rinsed five times (5 min/rinse) using sterile PBS. The scaffolds were implanted into SCID mice to monitor VEGF release when directly incorporated and, for all other studies, C57Bl/6J mice were used. At designated time points, scaffolds were retrieved and placed in a dispase (2.4 Units/mL)/collagenase (200 Units/mL) solution to digest any tissue attached to the implant. Scaffolds were analyzed using the γ counter following tissue digestion to determine the unreleased quantity of ^{125}I VEGF remaining in the scaffold. The dissolved tissue was also analyzed to quantify ^{125}I VEGF in the tissue that had infiltrated the implants. Tissue sections were obtained at various distances from the implant site, along with blood and liver samples. These tissues were analyzed for the presence of ^{125}I VEGF using the γ counter. Quantities of ^{125}I VEGF were converted to VEGF concentrations (ng/mL) using the known specific activity of the tracer and the weighed masses of the tissue samples (assuming tissue density of 1.0 g/mL). For all scaffolds, 3 μg of protein were incorporated. NIH guidelines for the care and use of laboratory animals (NIH publication No. 85–23, revised in 1985) have been observed.

Vessel formation

Scaffolds containing no growth factor and scaffolds directly incorporating 3 μg of VEGF were prepared as described earlier and subcutaneously implanted in C57Bl/6J mice (n = 3, per animal per condition), as previously described.[7–9] Scaffolds were retrieved from subcutaneous pockets after 2 weeks, fixed in formalin, and stored in ethanol. Tissues were subsequently embedded in paraffin, sectioned (~5 μm thick), and sections were placed on glass slides at the histology core in the School of Dentistry at the University of Michigan. The sections were stained for CD31 (antigen found on ECs) at the University of Michigan Cancer Center Histology Core, to identify blood vessels, as previously described.[7–9] The density of blood vessels was determined as previously described.[7–9] In brief, CD31-stained sections were viewed at 200× magnification using a light microscope (Nikon; Indianapolis, Indiana). Blood vessels in three different tissue samples from each condition (n = 3 per condition) were manually counted. The matrix area was determined using Image Pro Plus software to calculate the blood vessel density from the number and area measurements.

Statistical analysis

Student *t*-tests were performed on the raw data of the release studies and *in vivo* angiogenesis study. For the studies quantifying VEGF presence in the blood and liver, the statistical difference of these measured values from zero was determined.

RESULTS

Protein distribution in polymer scaffold

Both types of particles—used to fabricate the scaffolds (microspheres and particulate PLG)—foamed and fused together to create physically continuous structures in which neither distinct microspheres nor particulate PLG could be distinguished, as previously reported.[7–9] The distribution of protein incorporated into scaffolds with both methods, direct versus pre-encapsulation into microspheres used to form the scaffolds, was assessed using protein labeled with two different fluorophores and confocal microscopy. Direct incorporation of protein into scaffolds resulted in an even distribution of protein throughout the scaffolds, and a localization predominantly near the surface, as evidenced by a high concentration of the protein adjacent to the pores in the scaffolds (Fig. 1). In contrast, pre-encapsulation of protein into the microspheres used in the foaming process to fabricate scaffolds resulted in a more discrete localization, and the protein was more deeply embedded in the scaffold (Fig. 1).

In vitro release kinetics

The release kinetics for protein incorporated with both methods was subsequently analyzed using ^{125}I VEGF. Protein incorporated directly was released with

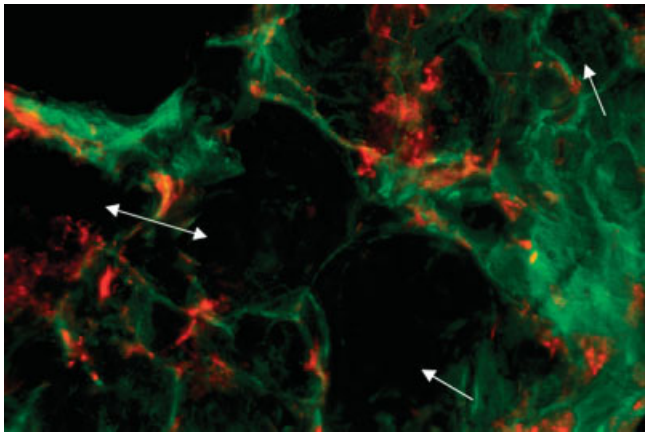

Figure 1. Confocal microscopy image of interior surface of scaffold formed with proteins containing either conjugated Alexa Fluor 488 using the direct incorporation method (positioned predominantly adjacent to pores), or Texas Red formed by pre-encapsulating the protein in 75:25 (MW 63 kDa) microspheres (embedded deeper in the matrix). Arrows denote open pores. Original magnification ×200. Note: Alexa Fluor 488 (green fluorescence) and Texas Red (red fluorescence). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

an initial rapid rate for the first 3–5 days, followed by a slower release rate for the remainder of the experiment duration. Protein release was sustained for more than 21 days. As the percentage of polymer in the form of microspheres used to prepare the scaffolds increased, the burst release (amount released in the first 24 h) increased from (25 ± 3)% (all 85:15 particles) to (54 ± 3)% (all microspheres) (Fig. 2).

The release kinetics of protein pre-encapsulated into microspheres before forming into scaffolds was next assessed to determine whether this approach would allow for a delayed release and to assess whether this release could be regulated by the PLG composition (lactide to glycolide ratio and molecular weight). ^{125}I VEGF was incorporated into microspheres of 85:15, 75:25, or 50:50 PLG. Scaffold protein release kinetics were similar from pre-encapsulation into microspheres of 85:15 and any of the low molecular weight 75:25 PLG. However, when delivering protein from microspheres made from 50:50 PLG, the release rate was slightly greater for the burst portion of the profile and faster during the sustained portion relative to the other two polymer compositions [Fig. 3(A)]. The effect of PLG molecular weight on the release kinetics was determined using 75:25 PLG, ranging from 16 to 98 kDa. The burst portion of the release profiles was similar for all three molecular weights, and only a slight difference was noted in the slopes of the sustained release rates [Fig. 3(B)]. However, the release rate of protein pre-encapsulated in PLG microspheres was, in all conditions, slower than that of the protein directly incorporated into scaffolds [compare Figs. 3(A,B) to Fig. 2].

The effects of several processing parameters on release kinetics when the protein was pre-encapsulated in microspheres were also analyzed. Specifically, the effects of polymer concentration in the fabrication of microspheres, the scaffold size, and the construction of the scaffold using all microspheres or a combination of microspheres and polymer particles, were analyzed. Increasing the PLG concentration in the microsphere preparation process from 5 to 30% halved the protein released during the burst from 11 to 5% (Fig. 4). However, the protein incorporation efficiency in the microspheres was decreased from (36 ± 5)% to (11 ± 0.4)%. Scaffolds of differing dimensions were prepared from the polymer in the form of all microspheres as well as from a combination of microspheres and 85:15 particulate. The protein release kinetics for the scaffolds formed entirely from microspheres with protein pre-encapsulated in 50:50 and 75:25 (16 and 63 kDa) PLG during the burst were only weakly affected by scaffold size, with smaller scaffolds yielding slightly faster release kinetics as the lactide concentration and molecular weight increased. In contrast, no differences were noted in the sustained release period.

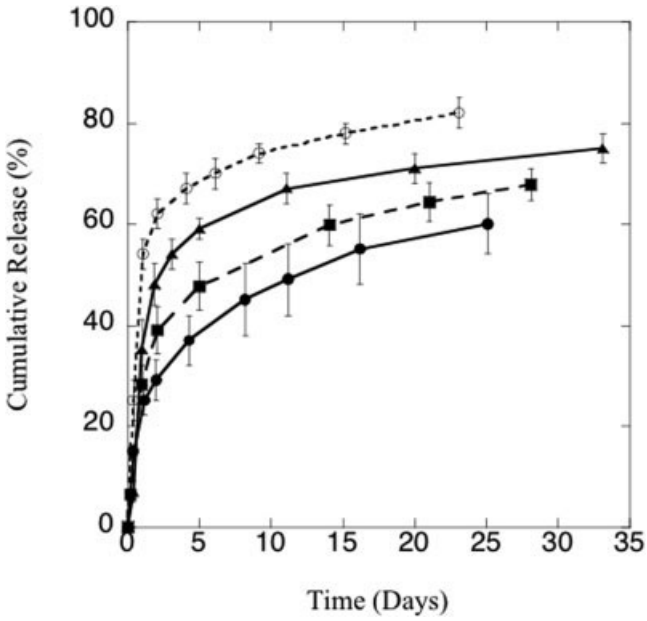

Figure 2. Release profiles of VEGF directly incorporated into scaffolds fabricated from all microspheres (open circles), a combination of 85:15 PLG particles and either 30% (solid triangles) or 20% (solid squares) microspheres, or all 85:15 particles (solid circles). Data represent average values ($n = 4–8$), and error bars indicate standard deviation, except for scaffolds with 20% microspheres in which error bars represent standard error of the mean. The differences in release values for scaffolds formed with all microspheres or 30% microspheres, as compared to all other conditions, were statistically significant ($p < 0.05$) at all time points. The differences between the scaffolds fabricated with 20% microspheres and scaffolds formed with no microspheres were not statistically significant ($p > 0.05$), except at day 2 and 5.

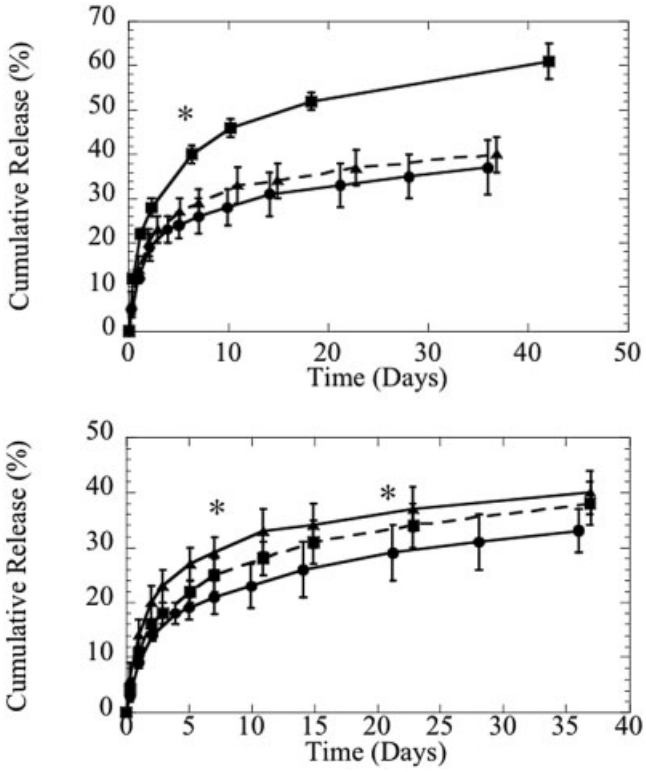

Figure 3. Effects of polymer composition and molecular weight on release of VEGF pre-encapsulated in microspheres. (A) Pre-encapsulated protein release from scaffolds formed from 85:15 PLG particles and microspheres fabricated using 50:50 PLG (solid squares), 75:25 (solid triangles), and 85:15 (solid circles). * denotes that differences in values from 50:50 PLG scaffolds, as compared to the other two conditions, were statistically significant ($p < 0.0001$) after day 3. No statistically significant differences in values between the 75:25 and 85:15 PLG conditions were noted ($p > 0.05$) at any time point. (B) Protein release rates from matrices formed with various 75:25 PLG [MW = 16 kDa (solid squares), 63 kDa (solid triangles), 98 kDa (solid circles)] microspheres. The differences between the values at each condition, as compared to the other conditions, were generally not statistically significant. The only exceptions were at day 7 (*), where $p < 0.01$ between all conditions, and at day 21 (*) for 16 and 63 kDa relative to 98 kDa 75:25 PLG ($p < 0.05$). Data represent average values ($n = 4–8$), and error bars indicate standard deviation.

Scaffolds fabricated entirely from 75:25 (MW 98 kDa) or 85:15 PLG microspheres demonstrated faster release rates during both the burst and the sustained portion of protein delivery (Table I). Finally, varying the ratios of microspheres versus particulate polymer—used to fabricate scaffolds—resulted in little effect on the burst release. The only exception was with 75:25 (MW 16 kDa) PLG, in which the scaffolds formed entirely from microspheres had a slightly higher burst release (Table I). During the sustained delivery period, using a mixture of microspheres and particulate—to form scaffolds—resulted in a slower protein release rate. The only exception was when 85:15 PLG was used to form microspheres, as no difference was evident in this condition (Table I).

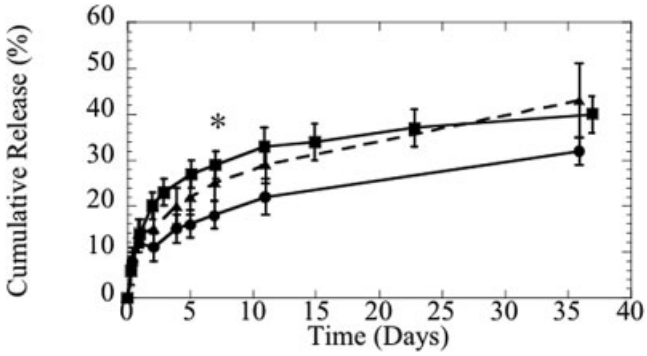

Figure 4. VEGF release from scaffolds prepared with 85:15 particulate PLG and protein pre-encapsulated in 75:25 (MW = 63 kDa) PLG microspheres. The concentration of polymer in the microscope preparation process was varied from 5% (solid squares) to 15% (solid triangles), and 30% (solid circles). Data represent average values ($n = 4–8$), and error bars indicate standard deviation. The differences in values between conditions were not statistically significant, with the exception of day 7 (*), at which the difference between each of the conditions compared to the others was significant ($p < 0.015$).

Protein release and localization *in vivo*

VEGF release kinetics and subsequent distribution throughout the surrounding tissues were next analyzed following scaffold implantation into the subcutaneous tissue of mice to determine how the release kinetics observed *in vitro* related to *in vivo* release.

TABLE I
VEGF Released in the First 24 h (Burst) and the Subsequent Release Rate from Scaffolds Prepared from Various PLG Types and Formulations

PLG type	Burst (%)	Subsequent Release (%/day)
Small[a] scaffolds (all microspheres)		
50:50	35	0.7
75:25 (16 kDa)	37	0.7
75:25 (63 kDa)	32	0.8
75:25 (98 kDa)	24	1.3
85:15	20	0.7
Large[b] scaffolds (all microspheres)		
50:50	22	0.8
75:25 (16 kDa)	24	0.8
75:25 (63 kDa)	17	0.9
75:25 (98 kDa)	10	0.6
85:15	7	0.4
Large[b] scaffolds (mixture[c])		
50:50	22	0.6
75:25 (16 kDa)	11	0.5
75:25 (63 kDa)	11	0.4
75:25 (98 kDa)	9	0.5
85:15	12	0.4

[a]Small: 4.7 × 3 mm^2 scaffolds.
[b]Large: 13 × 3 mm^2 scaffolds.
[c]Mixture: 85:15 particles + microspheres.

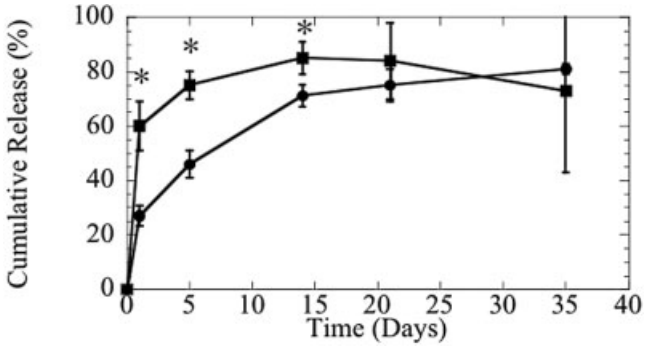

Figure 5. *In vivo* VEGF release profiles. VEGF directly incorporated into scaffolds (solid squares) or pre-encapsulated in 75:25 PLG (i.v. = 0.59 dL/g, 63 kDa) microspheres (solid circles). Data represent average values ($n = 5$), and error bars indicate standard deviation. * denotes significant differences ($p < 0.02$) between the values of the two conditions.

Scaffolds were removed at various time points, and the ^{125}I VEGF remaining in the polymer was quantified to determine the release kinetics of VEGF. Direct incorporation led to an initial burst release of ~60% of the total incorporated protein within the first 24 h, slightly greater than that observed *in vitro,* followed by a sustained release of 0.6% per day. VEGF pre-encapsulated in microspheres prior to scaffold fabrication led to a lower burst release (~27% of incorporated VEGF), followed by a greater release rate (1.8% per day) than those observed with VEGF directly incorporated into scaffolds (Fig. 5). The rate of protein release was greater *in vivo* in both the burst and subsequent release phases, as compared to that measured *in vitro*.

The distribution of VEGF released from the scaffolds was subsequently analyzed to determine whether a localized delivery was achieved with this system. Both the tissue that had grown into the scaffolds and surrounding tissues, along with the internal organs, were analyzed. At 24 h following implantation, a high VEGF concentration was found within the tissue growing into the scaffolds, and the VEGF concentration rapidly declined with distance from the scaffold (Fig. 6). The VEGF concentration within the tissue infiltrating the scaffold and in the surrounding tissues decreased at day 5 and further declined at day 21. However, at all time points, the VEGF concentration in the surrounding tissue was greater than 10 ng/mL. Blood samples drawn from the left ventricle of the heart showed the presence of trace quantities of protein (25 ± 33 ng/mL) at day 1, but only baseline quantities at the other time points [Fig. 7(A)]. Similarly, liver tissue contained very low concentrations of VEGF at day 1 (10 ± 2 ng/mL), and VEGF was undetectable at all later time points [Fig. 7(B)]. In both cases, at all time points, the values were not significantly different from zero ($p > 0.05$).

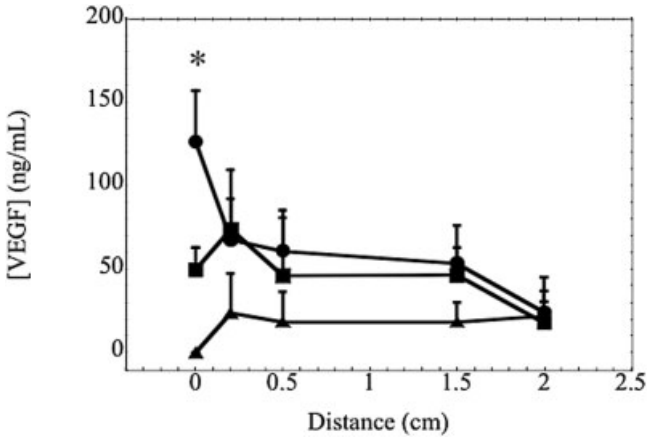

Figure 6. VEGF distribution in animal tissue sections around implant site. VEGF concentration both within the tissue invading scaffolds (distance = 0 cm) and within tissue sections at various distances from the implant site at day 1 (solid circles), 5 (solid squares), and 21 (solid triangles). Data represent average values ($n = 5$), and error bars indicate standard error of the mean. * denotes that the differences in values between the various time points was statistically significant ($p < 0.03$) at that distance.

New blood vessel formation

The utility of this system to qualitatively and quantitatively enhance local angiogenesis was next as-

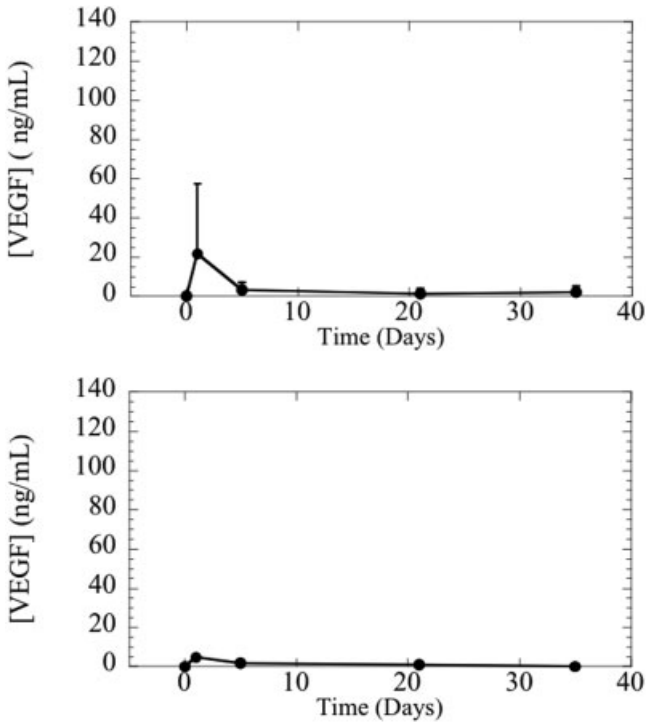

Figure 7. VEGF concentration measured in (A) blood samples taken from heart and (B) liver tissue measured over a 5-week period. Data represent average values ($n = 5$), and error bars indicate standard deviation. No values from either site at any time were statistically distinct ($p > 0.05$) from zero.

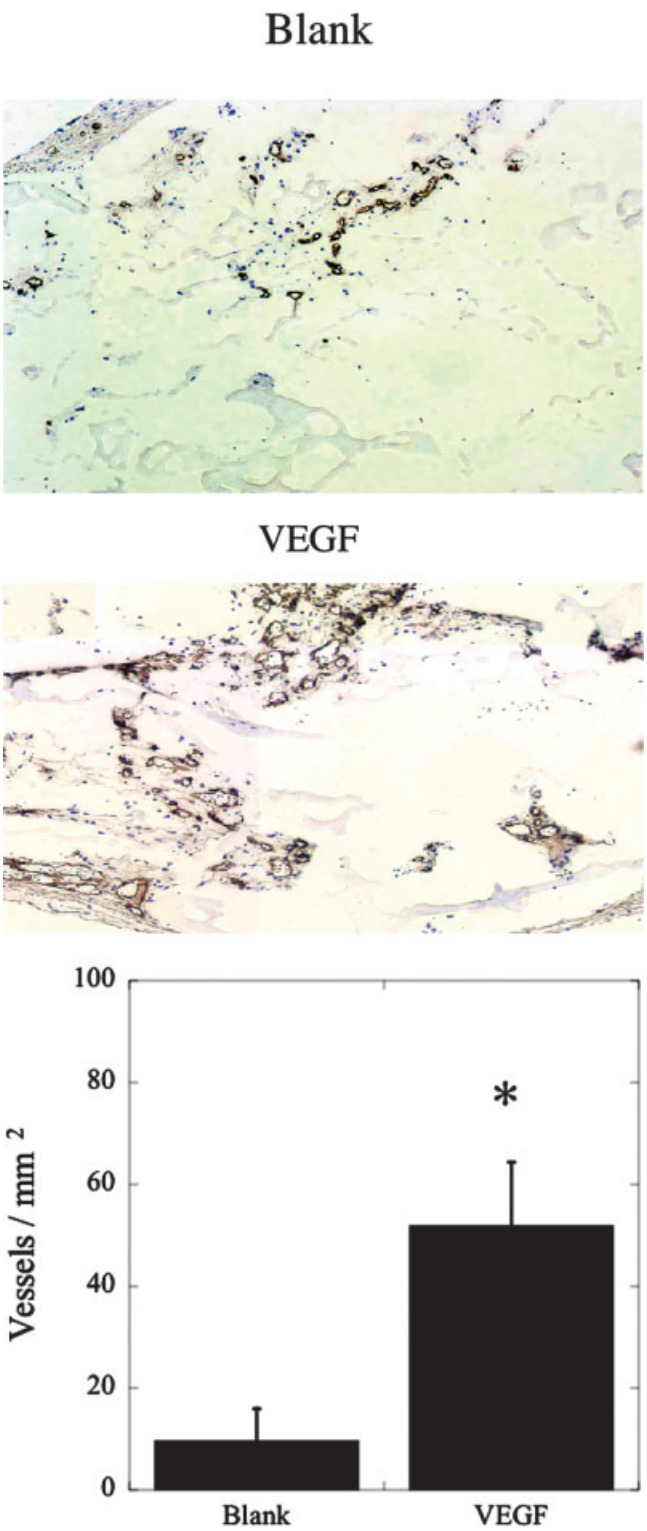

Figure 8. Vessel density produced in scaffolds containing no growth factor and those rapidly releasing VEGF, following 2 weeks of subcutaneous implantation. Photomicrographs of tissue sections from blank scaffolds (A) and VEGF-releasing scaffolds (B) following immunohistochemical staining for CD31 (EC marker) (×100 magnification). (C) Quantification of vessel density. Values represent averages and standard deviation, and * indicates statistically significant difference ($p < 0.015$) between the two conditions. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

sessed. Scaffolds releasing VEGF (direct incorporation) induced significant growth of microvessels relative to the control condition, as evidenced by the much larger density of CD31 positively stained vessels in histological samples [Fig. 8(A,B)]. The rapid release and a sustained localized presence of VEGF yielded a statistically significant increase in vessel density ($p < 0.05$) [Fig. 8(C)].

DISCUSSION

Directly incorporating VEGF versus pre-encapsulating the protein prior to incorporation into polymer scaffolds altered the distribution of VEGF in the scaffold and how the factor was released. Manipulation of polymer properties and processing parameters, such as polymer composition, scaffold size, and makeup, resulted in minor alterations in the release kinetics. Additionally, the protein release kinetics *in vivo* were also controlled by the mechanism of protein incorporation into scaffolds, and the released VEGF created a local concentration in tissue sections surrounding the implanted matrix, with little systemic exposure to the protein.

The manner of protein incorporation into the polymer matrix determined the distribution of the growth factor in the scaffold and affected its release. Analysis of the polymer constructs via confocal microscopy demonstrated that direct protein incorporation predominantly positioned the protein adjacent to the pores, potentially allowing it to diffuse rapidly out of the matrix. Pre-encapsulation of VEGF into microspheres subsequently used to form scaffolds led to the protein being more deeply embedded in the polymer, and this was reflected in the slower release as compared to protein directly incorporated into the matrix. In all studies investigating pre-encapsulated protein release, the rate of delivery was significantly slower than the release demonstrated with direct protein incorporation. The protein release rate was sensitive to certain, but not all polymer properties and processing parameters that were tested. The ratio of lactide to glycolide in the PLG affected the release rate of pre-encapsulated protein. However, weak effects on VEGF release kinetics were observed with alterations in polymer molecular weight over the tested range. In general, smaller scaffolds also had faster protein release kinetics than did larger counterparts with the same composition. Altogether, these data suggest that the method of protein incorporation had the greatest effect on release kinetics. However, the release kinetics achieved with the two incorporation techniques could be fine-tuned through alterations in a variety of polymer properties and processing parameters.

In all studies using pre-encapsulated VEGF *in vitro*,

the initial burst release was followed by an incomplete release. The burst release from these PLG scaffolds most likely resulted from the rapid desorption or diffusion of VEGF that associated with the surface of the microspheres during the fabrication process. The subsequent release most likely resulted from the protein located deeper within the microspheres that diffused through the polymer and/or was released as the polymer degraded. This phenomenon has been illustrated in several studies administering other bioactive factors[14–18] from 50:50 PLG microspheres. One of the primary sources of the incomplete or halted release has been attributed to protein instability.[19–23] The degradation of the polymer into lactic and glycolic acids generates an acidic microenvironment in which proteins readily experience hydrolysis (e.g. cleaved Asp-X linkages) and lose stability.[24–26] The hydrolyzed proteins may denature,[15] nonspecifically adsorb onto polymer surfaces, [27,28] and form noncovalent and covalent aggregates.[29] As the polymer degrades or erodes, new small pores develop, increasing the surface area available for protein to adsorb.[28] Additionally, the acidic microenvironment promotes alterations in the surface and internal morphology of the polymer, leading to the development of a "skin" layer on the microsphere surface.[30] This skin layer affects the permeability[30] of the polymer and the subsequent protein release after the burst, leading to the incomplete release. Although these previous studies were conducted on microspheres only, the findings most likely relate as well to polymer scaffolds prepared using microspheres. Methods to improve protein stability in PLG microspheres remain under development.[31]

The PLG scaffolds were successful in delivering VEGF *in vivo* with two distinct release kinetics, a rapid or a delayed release rate. The differences in release rates noted *in vitro* versus *in vivo* may be related to variations in protein stability in the two environments. As the polymer degrades, the acidic microenvironment generated *in vitro* may not exist *in vivo* because of the natural removal of waste products by the host and the maintenance of a physiological pH. In the *in vitro* experiments, the released protein may have formed covalent aggregates[29] and have nonspecifically adsorbed onto the surfaces of the matrix,[27,28] reducing the amount of protein measured in the release buffer and causing incomplete protein release. *In vivo*, many endogenous proteins exist that are free to adsorb onto the surface of the scaffold, perhaps blocking VEGF association or allowing the released protein to more fully disengage from the matrix or both. Additionally, the physical forces that exist in a mechanically dynamic setting may have increased the release of growth factors[32] relative to the mechanically static release environment *in vitro*. Two mice models, SCID and C57Bl/6J mice, were used in the *in vivo* studies. The use of the SCID mice in the first study investigating VEGF release following direct incorporation is not expected to affect the results, as an immune response requires several days to present, and so the presence or lack of immune competence in host animals should only be significant over longer time frames.

The VEGF released from the polymer matrix was distributed in tissue sections up to 2 cm from the implant, and these molecules remained localized to their target area with little systemic exposure, as evidenced by the low levels of VEGF detected in samples of blood and liver tissue. VEGF, once released from the scaffolds *in vivo*, is expected to be transiently present because of its short half-life. Therefore, the continuous high levels of VEGF in the tissues surrounding the implanted PLG are most likely dependent on continuous VEGF release from the scaffold. In addition, the very transient systemic VEGF exposure with this delivery system is unlikely to lead to angiogenesis at other sites, because of the lack of sustained VEGF presence anywhere except in the immediate vicinity of the implanted scaffold. Although the specific *in vivo* VEGF concentration required to elicit an EC response is unknown, the *in vitro* concentration of VEGF required to invoke an EC response is ~10 ng/mL.[8] The VEGF concentration in tissue samples, resulting from sustained VEGF delivery in this system, were equal to or greater than this value for the 21-day duration of the experiment. To perform this calculation, the density of tissue was assumed to be the same as water (~1.0 g/mL). Previous studies from our group, with both of these approaches to protein encapsulation, have demonstrated the biological activity of VEGF released from these polymer scaffolds.[8,9] Additionally, the significant increase in vessel density demonstrated in these *in vivo* studies confirms the bioactivity of the VEGF incorporated and released from this polymer system.

CONCLUSION

In summary, a PLG system with the ability to administer proteins in a temporal fashion was developed using two protein incorporation techniques. The incorporation techniques determined the distribution of the protein in the matrix and the kinetics of release. Coupling these incorporation methods with several manipulations of polymer properties and formulation variables allows one to alter the release. The released VEGF was present up to 2 cm from the implant and remained localized to the target area with negligible release into the systemic circulation. Released VEGF induced significant vessel formation in the scaffold tissue, demonstrating biological activity of the released protein. These results support the concept of

localized angiogenesis for therapeutic purposes, as well as the development and understanding of other processes that require temporal delivery of bioactive factors.

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