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Research Paper

Active Blood Vessel Formation in the Ischemic Hindlimb Mouse Model Using a Microsphere/Hydrogel Combination System

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Purpose. We hypothesize that the controlled delivery of rhVEGF using a microsphere/hydrogel combination system could be useful to achieve active blood vessel formation in the ischemic hindlimb mouse model, which is clinically relevant for therapeutic angiogenesis without multiple administrations.
Methods. A combination of poly(d,l-lactide-*co*-glycolide) (PLGA) microspheres and alginate hydrogels containing rhVEGF was prepared and injected intramuscularly into the ischemic hindlimb site of mouse model, and new blood vessel formation near the ischemic site was evaluated.
Results. The controlled release of rhVEGF from the combination system effectively protected muscles in ischemic regions from tissue necrosis. Interestingly, the number of newly formed, active blood vessels was significantly increased in mice treated with the rhVEGF-releasing combination system.
Conclusion. A microsphere/hydrogel combination system provided a useful means to deliver therapeutic angiogenic molecules into the body for the treatment of ischemic vascular diseases, which could reduce the number of administrations of many types of drugs.

KEY WORDS: ischemic hindlimb model; localized delivery; microsphere/hydrogel combination system; therapeutic angiogenesis; vascular endothelial growth factor.

INTRODUCTION

Current clinical trials to regenerate tissues such as blood vessels generally rely on surgical operation or bolus drug administration, which often requires either reoperation or repeated administration (1). Ischemic vascular diseases, such as cardiovascular disease and peripheral ischemic disease, cause critical rates of morbidity and mortality in the world (2). A hypoxic stress is induced in tissues when the supply of oxygen and nutrients through surrounding capillaries is insufficient, leading to cellular apoptosis. Angiogenesis is the initial process of new blood vessel formation, and plays a critical role in treatment of ischemic vascular disease. Recent studies have proven that various angiogenic factors (3), stem cells or endothelial precursor cells (4), and genes (5) could be useful for therapeutic angiogenesis (6–10). Recombinant human vascular endothelial growth factor (rhVEGF) acts as a mitogen to endothelial cells (11), and has been essential for treatment of ischemic disease via intracoronary and intravenous administration (12,13). Intramuscular injection of the VEGF-encoding gene has been used to induce therapeutic angiogenesis in the ischemic limb model (14).

Although angiogenic factors can significantly enhance the efficacy of angiogenesis *in vivo*, over-expression or over-dose of the factors may lead to various, unwanted side effects at normal sites (15). To successfully prevent overdosing, many delivery systems that control the release of therapeutics have been developed. One potential approach to treat patients with chronic vascular disease is to use a localized drug delivery system that can release drug molecules in a controlled manner at the desired site, using biocompatible and biodegradable polymeric systems. For example, hydrogels and micro- or nanoparticles have been extensively utilized to date, and have shown great potential in protecting protein or peptide drugs from enzymatic degradation, which allows drug activity in the body to be maintained for relatively long time periods (16). The use of hydrogels assures biocompatibility because of the structural similarity of the hydrogels to highly hydrated macromolecular-based structures in the body. A variety of natural or synthetic polymers have been utilized to form hydrogels for drug delivery into the body (17,18). Alginate, among many natural polymers, forms gels easily in conjunction with divalent cations such as calcium ions, and is frequently used for localized delivery of proteins or peptides (19). Poly(d,l-lactide-*co*-glycolide) (PLGA) has also been used for protein or peptide delivery in the form of nano- or microparticles, as it has excellent biocompatibility and controllable degradation behavior (20).

In general, hydrogels rapidly release hydrophilic drug molecules (e.g., protein), and polymeric micro- and nanoparticles are susceptible to phagocytosis by macrophages in the body. For example, both VEGF and bFGF are completely released from alginate gels within 7 days *in vitro* (21). To

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overcome these limitations, we previously developed microsphere/hydrogel combination systems, consisting of PLGA microspheres and alginate hydrogels, as an injectable tool for sustained and localized delivery of protein drugs (22–24). In this paper, we report *in vivo* efficacy of microsphere/hydrogel combination systems containing rhVEGF in treatment of ischemic vascular diseases using the hindlimb ischemia mouse model, which is clinically relevant to therapeutic angiogenesis. After hindlimb ischemia was induced in mice, a combination delivery system that can release rhVEGF in a sustained manner was injected into the ischemia region, and new active blood vessel formation near the ischemic site was evaluated.

MATERIALS AND METHODS

Preparation of Microsphere/Hydrogel Combination Systems Containing rhVEGF

PLGA (RESOMER® RG 502H, Boehringer Ingelheim) microspheres containing recombinant human vascular endothelial growth factor (rhVEGF, Peprotech Asia) were prepared using a water-in-oil-in-water (w/o/w) double emulsion method (25). Polymer and growth factor were dissolved in ethyl acetate (EA, Wako Pure Chemical Industries) and deionized water, respectively. The solutions were emulsified using a probe type sonicator (Branson Digital Sonifier®) for 30 s in an ice bath (0.25 μg/mg rhVEGF/PLGA, w/w). A 4% aqueous poly(vinyl alcohol) (PVA, MW 27,000–32,000) solution was poured into the emulsion (w/o), and the emulsion was emulsified again to form double emulsion (w/o/w) using a homogenizer (Ultra-Turrax® T25 basic, IKA®-Werke) for 5 min at 6,000 rpm. The double emulsion was poured into a 0.4% aqueous PVA solution (300 ml) and mixed thoroughly using a mechanical stirrer at 800 rpm for 3 h to evaporate the organic solvent. The solidified microspheres were washed with de-ionized water five times and lyophilized. A rhVEGF-loaded microsphere/hydrogel combination system with various mixing ratios (PLGA microsphere/alginate hydrogel, g/g) was prepared by ionic cross-linking of an alginate solution containing a suspension of the PLGA microspheres using calcium sulfate ($CaSO_4$) (24). The alginate gels without PLGA microspheres were also prepared. The total amount of rhVEGF was kept constant (10 μg rhVEGF/ml) in both combination system and alginate gel only (no microsphere).

In Vitro rhVEGF Release

The rhVEGF-loaded microsphere/hydrogel combination system was cut into disks (15 mm diameter and 2 mm thick) and washed with Dulbecco's phosphate-buffered saline (DPBS, Gibco). The disks were then placed in 12-well tissue culture plates (Corning), and DPBS was added (37°C, 5% CO_2 atmosphere). At predetermined time periods, the supernatant was removed and replaced with fresh DPBS. The amount of the released rhVEGF was determined by an rhVEGF ELISA assay kit (R&D).

Mouse Hindlimb Ischemia Model and Treatment

Hindlimb ischemia was induced in mice as previously described (26). Four-week-old, female athymic mice (20 g body weight, Orient Lab Animal) were anesthetized with rompun (20 mg/kg) and ketamine (80 mg/kg). The femoral artery and its branches were ligated through a skin incision using 6–0 silk suture (Ethicon). The external iliac artery and all of the above arteries were then ligated. The femoral artery was excised from its proximal origin as a branch of the external iliac artery to the distal point where it bifurcates into the saphenous and popliteal arteries. All the procedures were in compliance with Hanyang University Guidelines for the care and use of laboratory animals. Following arterial dissection, athymic mice were randomly assigned to an experimental group ($n=7$ for each group). Mice treated with PBS only after induction of hindlimb ischemia were used as a control. Arterial-dissected mice were treated with either alginate gels only (no rhVEGF), bolus rhVEGF injection, rhVEGF-loaded alginate gel (no microsphere), or rhVEGF-releasing combination system (mixing ratio=1, w/w) by intramuscular injection into the gracilis muscle in the medial thigh (1 μg rhVEGF/mouse). In order to assess neovascularization after treatment, 5-bromo-2′-deoxyuridine (BrdU, 25 mg/kg body weight, Sigma) was administered every other day for four weeks following the operation.

Histological and Immunohistochemical Analysis

Ischemic limb muscles were retrieved 28 days after treatment, embedded into an optimal cutting temperature compound (TISSUE-TEK® O.C.T. compound, Sakura Finetek), frozen, and cut into 10 μm-thick sections at −20°C. The tissue sections were stained with hematoxylin and eosin (H&E) to examine muscle degeneration and tissue inflammation. Masson's trichrome staining was performed to assess tissue fibrosis in the ischemic regions. To detect newly formed vessels in the ischemic region, tissue sections were immunofluorescently stained with anti-BrdU antigen (1:200 dilution, Sigma). Capillaries and arterioles in the ischemic regions were immunefluorescently stained with anti-von Willebrand factor (vWF, 1:500 dilution, Abcam) and anti-smooth muscle (SM) α-actin (1:500 dilution, Abcam), respectively. The presence of BrdU and other molecules (vWF and SM α-actin) were visualized with rhodamine- and FITC-conjugated secondary antibodies (1:50, Jackson ImmunoResearch Laboratories), respectively. The tissue sections were counterstained with DAPI, and then examined using a confocal microscope (LSM510, Carl Zeiss).

Western Blot Analysis

Tissue sections obtained from the ischemic hindlimb 28 days after treatment were homogenized using a Dounce homogenizer (50 strokes, 4°C) in ice-cold lysis buffer (15 mM Tris HCl, pH 8.0, 0.25 M sucrose, 15 mM NaCl, 1.5 mM $MgCl_2$, 2.5 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 2 mM NaPPi, 1 μg/ml pepstatin A, 2.5 μg/ml aprotinin, 5 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.125 mM Na_3VO_4, 25 mM NaF and 10 μM lactacystin). Protein concentrations of the homogenates were determined by the micro-bicinchoninic acid method (Pierce). Western blot analysis was carried out with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After transfer to an Immobilon-P membrane (Millipore), the proteins were probed with polyclonal antibodies against platelet endothelial cell adhesion molecules (PECAM, Abcam), followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1:5000 dilution, Santa Cruz

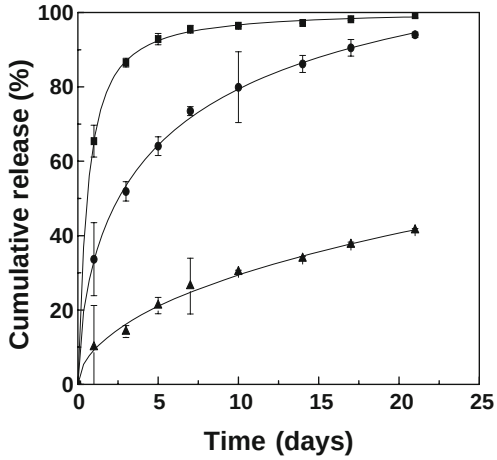

Fig. 1. *In vitro* rhVEGF release from combination delivery systems prepared at various mixing ratios (*filled square*, PLGA/alginate (w/w)=0; *filled circle*, 1; *filled upright triangle*, 2).

Biotechnology) for one hour at room temperature. The blots were developed using an enhanced chemiluminescence detection method (Amersham Bioscience). The band intensity on the western blot was quantified by densitometric scanning using Image-Pro Plus software (Media Cybernetics).

Statistical Analysis

All data are presented as the mean±standard deviation. Statistical analysis was performed using Student's *t*-test. Values of $*P<0.05$, $**P<0.01$, and $***P<0.001$ were considered statistically significant.

RESULTS

In Vitro rhVEGF Release from Combination Systems

Combination delivery systems were prepared at various mixing ratios of microspheres and hydrogels, and the release behavior of rhVEGF from the combination systems was monitored *in vitro*. The total concentration of rhVEGF was kept constant for all combination systems used in this study (94.2 ng/disk), with a loading efficiency of approximately 90%. The release of rhVEGF from alginate gels was almost complete within 5 days (mixing ratio=0), and the controlled release of rhVEGF from combination systems as a function of mixing ratios was achieved for three weeks (Fig. 1). The release behavior of rhVEGF from combination systems was dependent on the amount of rhVEGF-loaded microspheres in the system. The release rate of rhVEGF from the combination systems with mixing ratios of 1 and 2 were 2.3 and 1.5 %/day^{-1}, respectively.

Representative Photographs and Histological Analysis

Representative photographs of mice were captured at 7, 14 and 28 days after treatment (Fig. 2). The control group showed rapid and spacious necrosis in the ischemic hindlimb, resulting in complete limb loss by 28 days (Fig. 2a). All mice treated with a single injection of rhVEGF (Fig. 2b) and the rhVEGF-loaded alginate gels (no microspheres) (Fig. 2c) showed limb loss or severe limb necrosis, with significantly reduced numbers of salvaged limb. Intramuscular injection of the rhVEGF-loaded combination system significantly reduced the rate of limb loss compared to the other groups (Fig 2d). Most mice treated with the combination system exhibited a significantly enhanced limb salvage rate or mild limb necrosis with minimal limb loss (Table I).

Histological analysis was performed using H & E and Masson's trichrome staining methods, and revealed prominent muscle protection from ischemic damage in the rhVEGF-loaded alginate gel (Fig. 3c) and combination system groups (Fig. 3d). Tissue sections stained with H & E showed massive muscle degeneration and infiltration of numerous granulocytes and neutrophils in the control group (Fig. 3a). The rhVEGF single injection was not effective in preventing muscle degeneration in the mouse model (Fig. 3b). Masson's

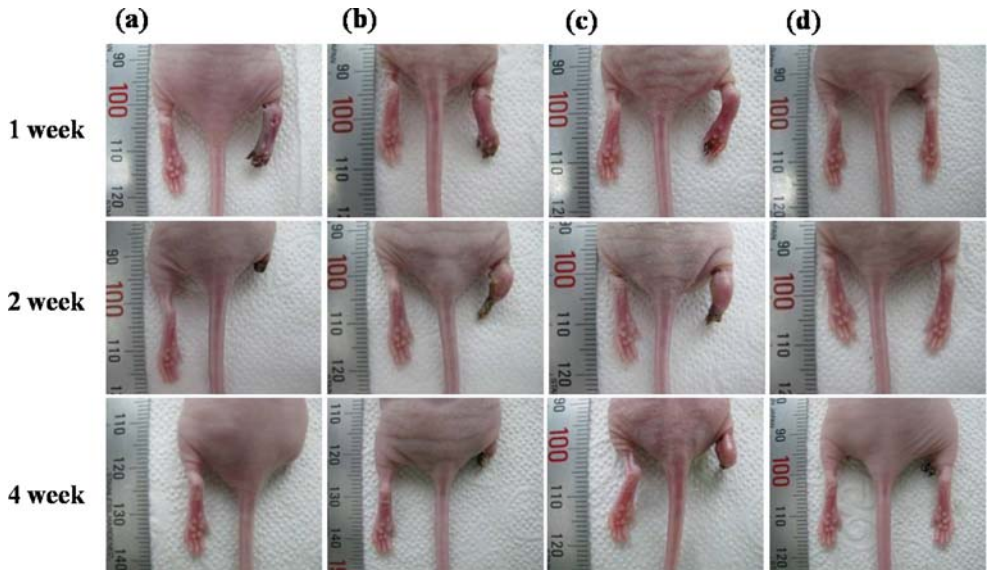

Fig. 2. Representative photographs of an ischemic hindlimb treated with (**a**) PBS (control), (**b**) rhVEGF single injection, (**c**) rhVEGF-loaded hydrogel, and (**d**) rhVEGF-loaded combination system.

Table I. Scoring of Ischemic Limb Four Weeks After Treatment (n=7)

	Control	rhVEGF Single Injection	rhVEGF-Loaded Alginate Gel	rhVEGF-Loaded Combination System
Limb salvage[a]	0	0	2	6
Foot necrosis[b]	2	3	2	1
Limb loss[c]	5	4	3	0

[a] Number of mice that have sound limbs with or without mild toe necrosis
[b] Number of mice that have severe foot necrosis with total loss of toes
[c] Number of mice that have no limb in ischemic regions

trichrome staining showed the same results as H & E staining. Muscle fibrosis in mice treated with rhVEGF-loaded alginate gels and the rhVEGF-releasing combination system was significantly attenuated, compared to other experimental groups.

Immunohistochemical Analysis

Localized and sustained delivery of rhVEGF using a combination system enhanced therapeutic angiogenesis in the mouse hindlimb ischemia model. Immunofluorescent staining

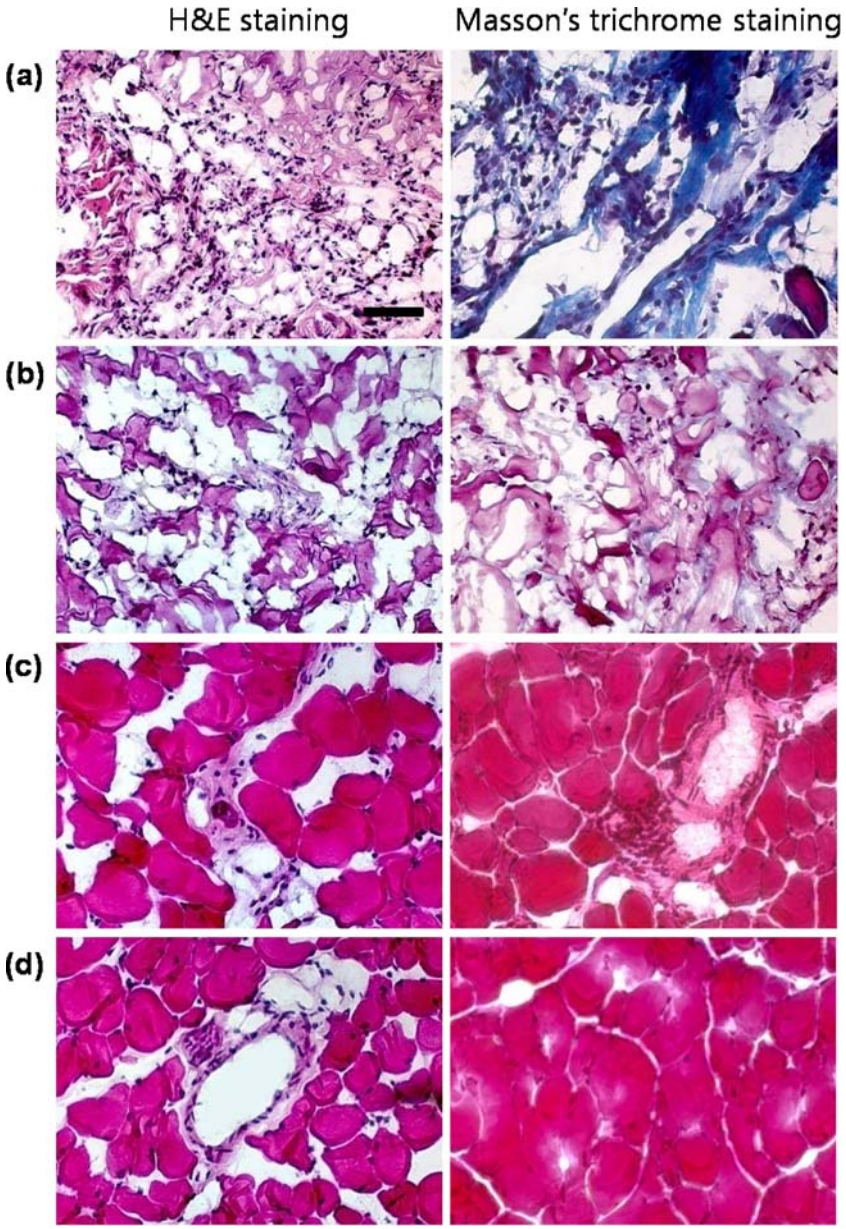

Fig. 3. H&E and Masson's trichrome staining of ischemic tissue sections retrieved from mice treated with (**a**) PBS (control), (**b**) rhVEGF single injection, (**c**) rhVEGF-loaded alginate hydrogel, and (**d**) rhVEGF- loaded combination system (scale bar, 20 μm).

for mouse smooth muscle (SM) α-actin (Fig. 4) and quantification of the arteriole density (Fig. 6a) revealed that arteriole formation was significantly enhanced in the combination system group (25.1±5.0/mm^2), when compared to the rhVEGF-loaded alginate gel group (13.0±2.6/mm^2). Immunofluorescent staining for mouse von Willebrand factor (vWF) (Fig. 5) and quantification of the capillary density (Fig. 6b) demonstrated that the combination system greatly enhanced capillary formation (205.8±78.2/mm^2), as compared with the rhVEGF-loaded alginate gel group (115.3±25.7/mm^2). Angiogenesis in mice treated with a single injection of rhVEGF was similar to the control group.

Near to the capillaries and arterioles in ischemic regions, BrdU-positive cells were found, and the cells were incorporated with newly formed mouse microvessels (Figs. 4 and 5). In mice treated with an rhVEGF single injection, however, few BrdU-positive microvessels were observed. Surprisingly, many BrdU-positive microvessels were found in mice treated with the combination system (Figs. 4, 5 and 6).

Western Blot Analysis

We also tested whether the combination system injected into the mouse hindlimb ischemia region could induce mature, active microvessels. Twenty-eight days after hindlimb ischemia induction, the combination system-treated group showed prominent expression of PECAM, indicating functional microvessel formation (Fig. 7). Mice treated with a single injection of rhVEGF and rhVEGF-loaded alginate gels showed significantly reduced PECAM expression, as compared to the combination system.

DISCUSSION

Systemic delivery of the VEGF gene or protein for therapeutic angiogenesis in ischemic hindlimb models has been frequently reported (27). However, these VEGF systems may cause side effects, such as hypotension or edema formation at tissues adjacent to ischemic regions by regulating abnormal VEGF expression (28). To overcome these limitations, local delivery systems such as micro/nanoparticles and hydrogels have been widely investigated. Localized and sustained delivery of bFGF using PLGA nanoparticles was useful for enhancing angiogenesis in animals (29). Relatively large amounts of bFGF-containing nanoparticles were required, however, as the nanoparticles were liable to be removed by macrophages. Biodegradable collagen hydrogels containing VEGF were also used to induce angiogenesis *in vivo* (30). Heparin hydrogels, functionalized with N-hydroxysuccinimidyl ester of poly(ethylene glycol)-*bis*-butanoic acid (SBA-PEG-SBA), were used to control the release behavior of VEGF (31). Microparticle-loaded polymeric scaffolds or hydrogels have also shown potential for sustained delivery of angiogenic molecules (32). However, few studies have been reported regarding the *in vivo* efficacy of combination delivery systems in therapeutic angiogenesis.

Although alginate hydrogels have often been exploited as an injectable tool for delivery of angiogenic factors for treatment of cardiovascular disease (33), angiogenic factors are released from the hydrogels within relatively short time periods. For this reason, combination delivery systems composed of both microspheres and hydrogels were fabricated by embedding PLGA microspheres into alginate gels. The combination systems were useful in controlling the release behavior of several proteins, including bovine serum albumin (BSA), heat shock protein 27 (HSP27), and rhVEGF (22–24). A microsphere/hydrogel combination system containing rhVEGF showed potential in promoting granulation tissue and blood vessel formation when the system was delivered into the subcutaneous tissue of mice (24). However, insufficient blood vessels were formed when rhVEGF-encapsulated PLGA microspheres were used without hydrogels.

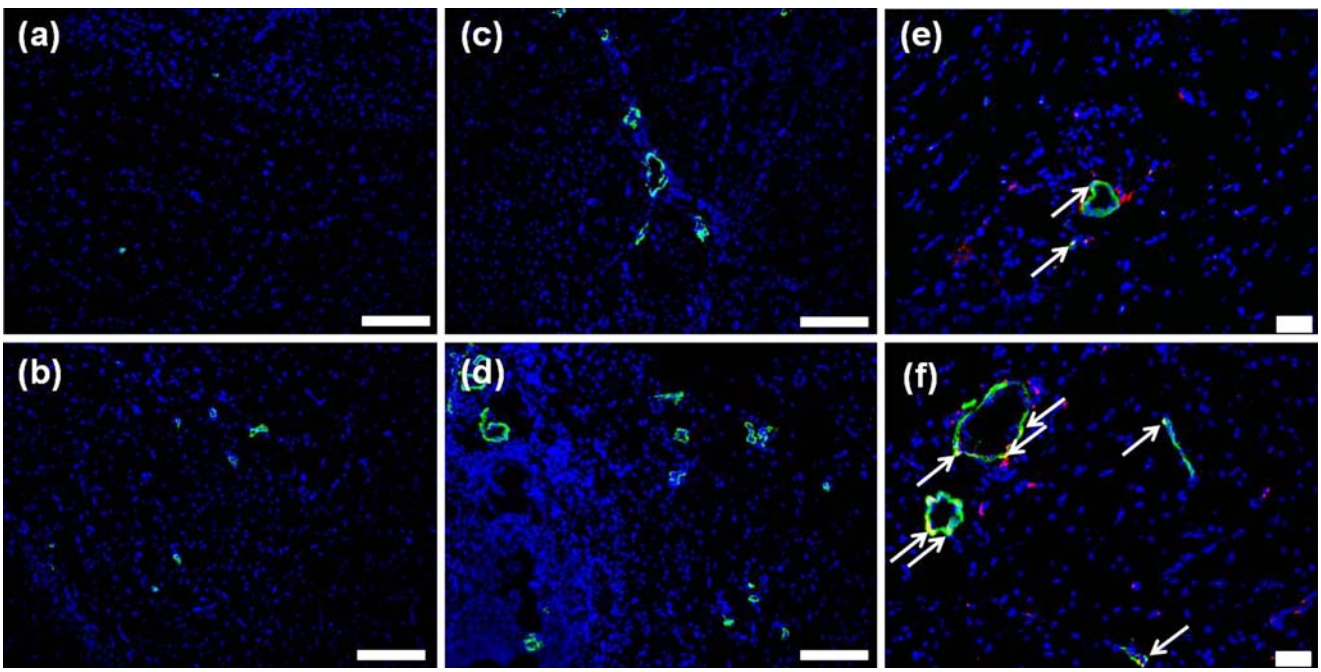

Fig. 4. Immunohistochemical staining for SM α-actin in ischemic tissues treated with (**a**) PBS (control), (**b**) rhVEGF single injection, (**c** and **e**) rhVEGF-loaded alginate hydrogel, and (**d** and **f**) rhVEGF-loaded combination system. Blue, green and red colors represent DAPI, SM α-actin, and BrdU, respectively. Arrows indicate newly formed arterioles (scale bar, 20 μm).

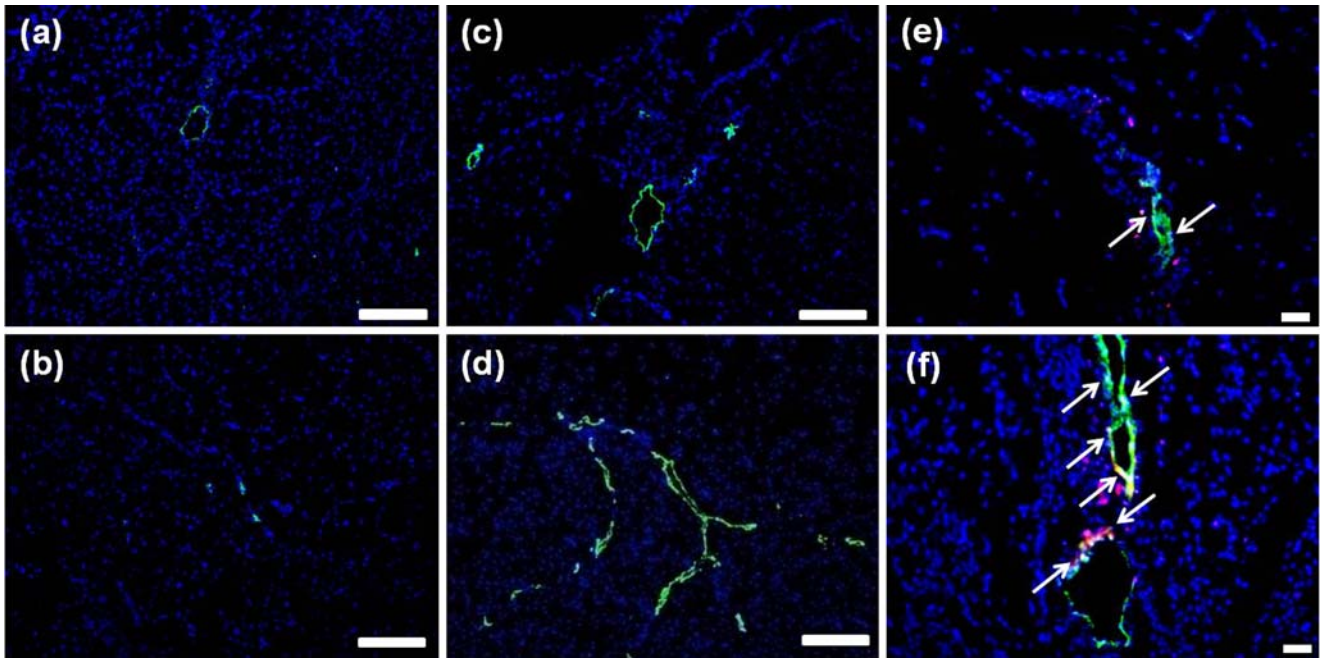

Fig. 5. Immunohistochemical staining for vWF in ischemic tissues treated with (**a**) PBS (control), (**b**) rhVEGF single injection, (**c** and **e**) rhVEGF-loaded alginate hydrogel, and (**d** and **f**) rhVEGF-loaded combination system. Blue, green and red colors represent DAPI, vWF, and BrdU, respectively. Arrows indicate newly formed capillaries (scale bar, 20 μm).

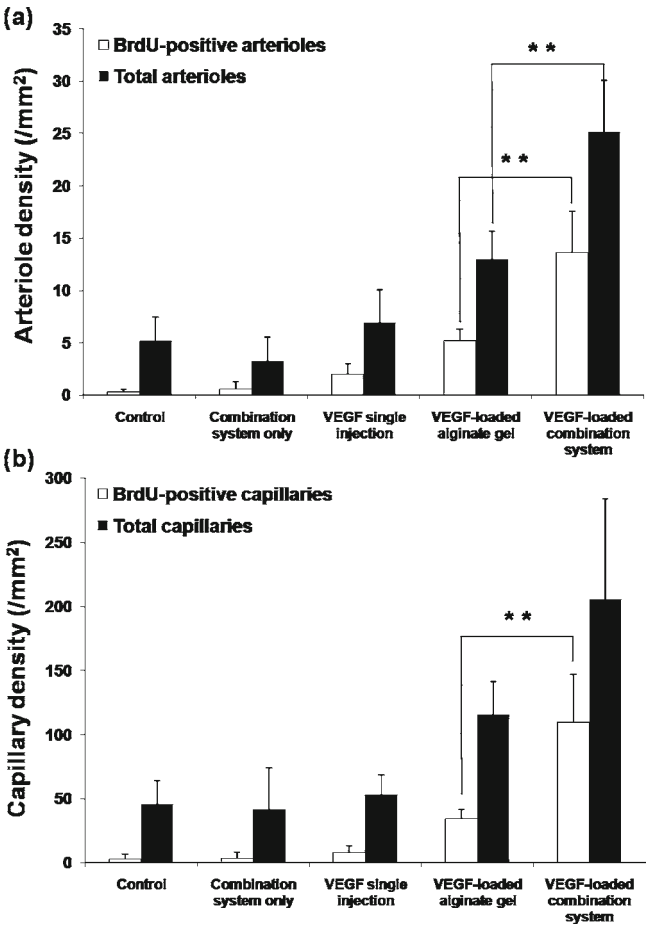

Fig. 6. Quantification of (**a**) arterioles and (**b**) capillary density in ischemic regions.

We demonstrated that a combination system was useful in inducing angiogenesis in the ischemic hindlimb mouse model. The *in vitro* release behavior of rhVEGF was dependent on the mixing ratio of microspheres and hydrogels in the combination delivery system. The sustained release of

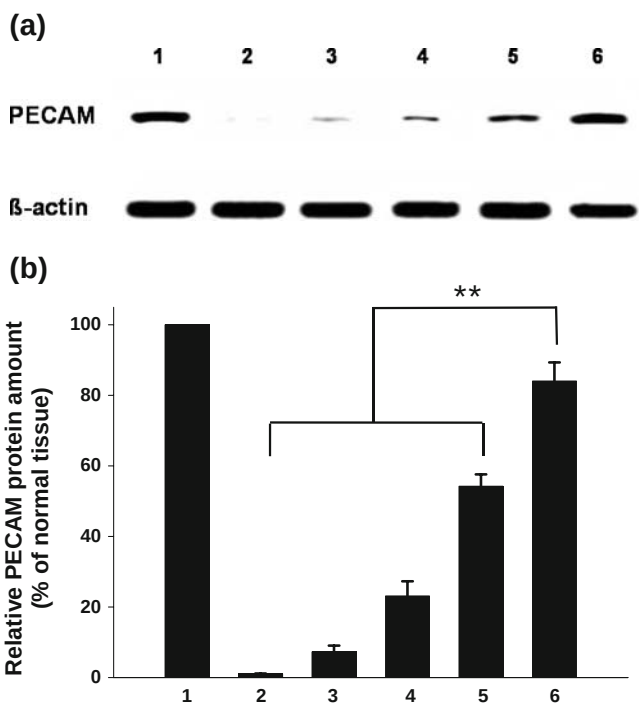

Fig. 7. (**a**) Western blot analysis for PECAM and (**b**) quantitative comparison of the band intensity (lane 1, normal tissue; lane 2, PBS; lane 3, combination system only; lane 4, rhVEGF single injection; lane 5, rhVEGF-loaded alginate hydrogel; lane 6, rhVEGF-loaded combination system).

rhVEGF from the combination system was achieved *in vitro*, and the mice were treated with the combination system after ischemic hindlimb induction (mixing ratio=1). Histological examinations revealed that tissues were protected from ischemic stress by the controlled release of angiogenic factors. Although growth factor-releasing alginate gels preserved muscles in the ischemic hindlimb region, new blood vessel formation was not effective, likely due to the rapid depletion of the factors from the gels as shown in Fig. 1. BrdU is commonly used to detect proliferative cells, as it can incorporate into the deoxyribonucleic acid of newly replicated cells. BrdU antibody was used to identify newly formed blood vessels (34). The number of BrdU positive blood vessels increased much more in the group treated with the rhVEGF-releasing combination system, as compared with alginate gels loaded with rhVEGF. This finding indicates that an rhVEGF-loaded combination system induces therapeutic angiogenesis much more effectively than rhVEGF-loaded alginate gels, due to the controlled release of the factors from the system. We also tested expression of PECAM as a marker of functional vessels in ischemic regions (35), and found expression was significantly enhanced when mice were treated with the combination system. In summary, we found that active new blood vessels *in vivo* were effectively produced by controlling the release of angiogenic factors using a combination delivery system.

CONCLUSION

We developed controlled drug delivery systems consisting of PLGA microspheres and alginate hydrogels, to promote active blood vessel formation *in vivo*. Microsphere/hydrogel combination delivery systems provided a useful means of delivering angiogenic factors in a sustained manner. The controlled release of rhVEGF from the combination systems protected muscles adjacent to ischemic sites from ischemic stress, and effectively regenerated active new arterioles and capillaries *in vivo*. This approach to controlling the release behavior of angiogenic molecules using microsphere/hydrogel combination delivery systems is useful to treatment of ischemic vascular diseases, as well as in many drug delivery applications, by reducing the number of administrations of many types of medication.

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