

Research Paper

Local and Sustained Vascular Endothelial Growth Factor Delivery for Angiogenesis Using an Injectable System

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Purpose. We hypothesize that a microsphere/hydrogel combination system could be useful for the local and sustained delivery of recombinant human vascular endothelial growth factor (rhVEGF) to enhance angiogenesis *in vivo*.

Methods. Poly(*d,l*-lactide-*co*-glycolide) (PLGA) microspheres containing rhVEGF were loaded into alginate gels by ionic cross-linking. The rhVEGF release from the system was monitored and bioactivity was tested *in vitro*. The combination system was subcutaneously injected into mice using a syringe, and new blood vessel formation was evaluated.

Results. Sustained rhVEGF release from the combination system was observed for 3 weeks, and the released rhVEGF remained bioactive. Endothelial cell proliferation was significantly enhanced when cells were cultured with the rhVEGF-releasing combination system *in vitro*. When the combination system was implanted, the granulation tissue layer was thicker with more newly formed blood vessels than that with a single dose VEGF injection.

Conclusion. The rhVEGF release was controlled by varying relative portions of microspheres and hydrogels in combination delivery systems, which efficiently promoted new blood vessel formation *in vivo*. This combination system could be a promising delivery vehicle for therapeutic angiogenesis.

KEY WORDS: angiogenesis; combination system; injectable; localized delivery; vascular endothelial growth factor.

INTRODUCTION

Ischemic vascular diseases have recently become a leading cause of morbidity and mortality in the world (1). Tissues require oxygen and nutrients supplied through the surrounding capillaries. Insufficient supplies induce hypoxia, leading to cellular apoptosis. To date, surgical operations have been frequently performed to treat ischemic vascular diseases (2,3), however, limitations remain such as thrombosis, limited durability, and re-operation (4). Angiogenesis promotes capillary sprouting and extension from preexisting blood vessels, leading to new blood vessel formation (5). Various angiogenic molecules, stem cells or endothelial precursor cells, and genes have been utilized to treat ischemic vascular diseases (6–9). In addition, recent studies report that angiogenesis is useful for tissue engineering applications, especially for large tissue formation (10–12).

Vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF), angiopoietin-1, and angiopoietin-2 have been reported as potent angiogenic molecules (13–15). The VEGF family is one of the most potent angiogenic regulators, comprised of VEGF-A, VEGF-B,

VEGF-C, and VEGF-D (16). Recombinant human vascular endothelial growth factor (rhVEGF) has 165 amino acids, and its bioactivity can be maintained by forming a dimer via disulfide linkages. Gene therapy can induce localized VEGF expression in ischemic myocardium for therapeutic angiogenesis (17). VEGF-B and its receptor VEGF-R1 promote angiogenesis in an *in vivo* ischemic hindlimb model by activating the Akt and eNOS-related pathway (18). Intracoronary rhVEGF administration was useful to treat patients with coronary artery disease (19).

VEGF is subject to enzymatic degradation and can be cleared from the body within comparatively short time periods, leading to repeated administration (20). For this reason, many drug delivery systems such as films, hydrogels, and nano- and microparticles have been used to control growth factor release and protect them from degradation (21). Hydrogels contain high water content and do not dissolve or dissociate, but maintain a three-dimensional structure in physiological conditions (22). Alginate gels have been widely used as an injectable tool to deliver various drug molecules (23). However, alginate gels rapidly release hydrophilic drug molecules during the initial stage. Polymeric micro- and nanoparticles have also been utilized to deliver bioactive molecules, including VEGF (24). These particles do not stay at the desired site without any supporting systems and are susceptible to phagocytosis by macrophages. In this study, we prepared a combination delivery system that consists of poly(*d,l*-lactide-*co*-glycolide) (PLGA) micro-

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spheres and alginate hydrogels containing rhVEGF. The rhVEGF release from the combination delivery system was investigated by varying mixing ratios between microspheres and hydrogels. Bioactivity of the released rhVEGF was tested using endothelial cells *in vitro*. We also tested if the rhVEGF-loaded combination system could enhance angiogenesis efficiently *in vivo* by subcutaneous injection into the back of mice, and the thickness of the granulation tissues and the number of newly formed blood vessels in the tissues were evaluated.

MATERIALS AND METHODS

Materials

Sodium alginate was purchased from FMC biopolymers (MW 200,000–300,000), and RESOMER[®] RG 502H (MW 10,000, 0.16–0.24 dl/g) was purchased from Boehringer Ingelheim. Recombinant human vascular endothelial growth factor (rhVEGF) was purchased from Peptrotech Asia. Poly (vinyl alcohol) (PVA, MW 27,000–32,000), calcium sulfate (CaSO₄), dimethyl sulfoxide (DMSO), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma-Aldrich. Dulbecco's phosphate buffered saline (DPBS) was purchased from Gibco. Ethyl acetate (EA) was purchased from Wako Pure Chemical Industries. Immunodeficient mice (BALB/c nude mice, female, 5 week-old) were supplied by Orient Bio Inc.

Preparation of Microsphere/Hydrogel Combination Systems Containing rhVEGF

PLGA microspheres containing rhVEGF were prepared using a water-in-oil-in-water (*w/o/w*) double emulsion method (25). Briefly, PLGA and rhVEGF were dissolved in EA and

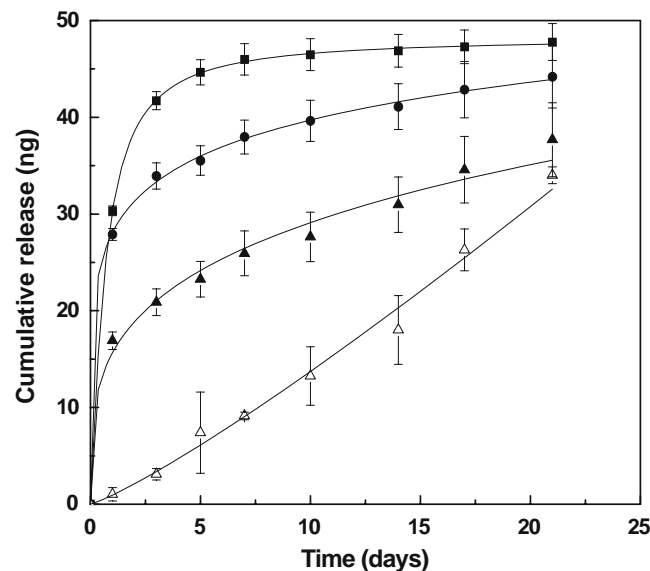


Fig. 1. *In vitro* rhVEGF release from microsphere/hydrogel combination delivery systems prepared at different mixing ratios (closed squares 0; closed circles 0.5; closed triangles 1.0; PLGA/alginate = *w/w*). The VEGF release from a VEGF-loaded PLGA microsphere suspension in DPBS was also monitored (open triangles).

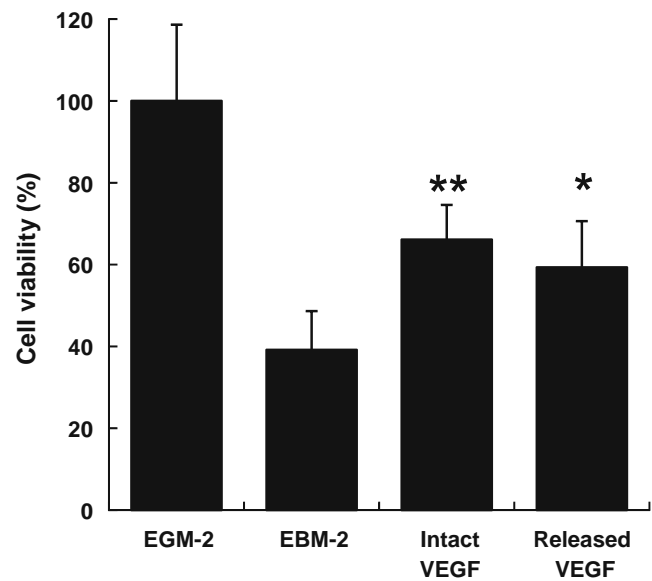


Fig. 2. Effect of VEGF released from the combination delivery system on HUVEC viability *in vitro* ([VEGF]=10 ng/ml; * $P < 0.05$, ** $P < 0.01$ vs. control (EBM-2); $n = 6$).

deionized water, respectively. The solutions were emulsified using a probe type sonicator (Branson Digital Sonifier[®]) for 10 s. The resulting emulsion (*w/o*) was poured into 4% aqueous PVA solution and emulsified again using a homogenizer (Ultra-Turrax[®] T25 basic, IKA[®]-Werke) for 5 min at 6,000 rpm (*w/o/w*). The obtained double emulsion was poured into 0.4% aqueous PVA solution (300 ml) and mixed thoroughly using a mechanical stirrer at 800 rpm for 3 h. The solidified microspheres were washed with de-ionized water five times and lyophilized. A microsphere/hydrogel combination system was prepared by ionically cross-linking a suspension of PLGA microspheres in an alginate solution using calcium sulfate (26). The mixing ratio of PLGA microspheres to alginate gel was varied from 0 to 1.0 (*w/w*).

In Vitro rhVEGF Release

Different amounts of PLGA microspheres were loaded into alginate hydrogels. The total rhVEGF in the system was kept constant by adding rhVEGF to the alginate solution

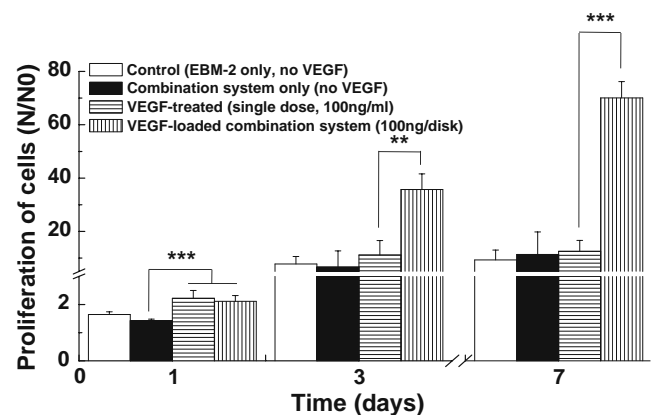


Fig. 3. Effect of VEGF released from the combination system on HUVEC proliferation *in vitro* (** $P < 0.01$, *** $P < 0.001$; $n = 6$).

Table I. HUVEC Growth Rates Cultured Under Various Conditions

Condition	Growth rate (day ⁻¹)
Control (no growth factor, no delivery system)	0.39±0.11
Combination system only (no VEGF)	0.40±0.16
VEGF treatment (single dose, 100 ng/ml)	0.47±0.07
VEGF-loaded combination system (100 ng/disk)	0.90±0.04

(47.1 ng rhVEGF/disk). The gels were cut into disks (15 mm diameter and 2 mm thick) and washed with DPBS. The rhVEGF-loaded combination systems were placed in 12-well tissue culture plates (Corning), and DPBS was added (37°C, 5% CO₂ atmosphere). At predetermined time intervals, the supernatant was removed and replaced with fresh DPBS. The amount of released rhVEGF was determined by an rhVEGF ELISA assay kit (R&D).

Bioactivity of Released rhVEGF

Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial growth media (EGM-2) (Clonetics). For all experiments, cells from passage two to five were used. Cell viability was evaluated by MTT assay. Endothelial cells were seeded onto 96-well tissue culture plates and incubated for 12 h at 37°C under 5% CO₂ atmosphere. Endothelial basal media (EBM-2) containing either intact or released rhVEGF was added to each well and incubated for 24 h. The cells were then washed with DPBS, and a solution of fresh media (180 µl) and MTT (20 µl, 0.5 mg/ml) was added to each well. The cells were incubated for 3 h at 37°C under 5% CO₂ atmosphere. Formazan crystals were dissolved by adding DMSO, and absorbance was measured at 540 nm with a spectrophotometer (SpectraMax M2^c).

In Vitro HUVEC Proliferation

Endothelial cells were seeded onto 12-well tissue culture plates, incubated for 12 h at 37°C under 5% CO₂ atmosphere, and washed with DPBS twice. Combination systems containing 100 ng rhVEGF/disk were then placed in the upper insert of transwell (Corning Incorporated Costar[®]), and the cells were cultured for 7 days. At each time point, the number of cells was counted by MTT assay.

In Vivo Angiogenesis

The combination delivery system (100 µl) containing 10 µg/ml rhVEGF was subcutaneously injected into the dorsal site of immunodeficient mice using a syringe. In brief, animals were anesthetized by an intramuscular injection of ketamine

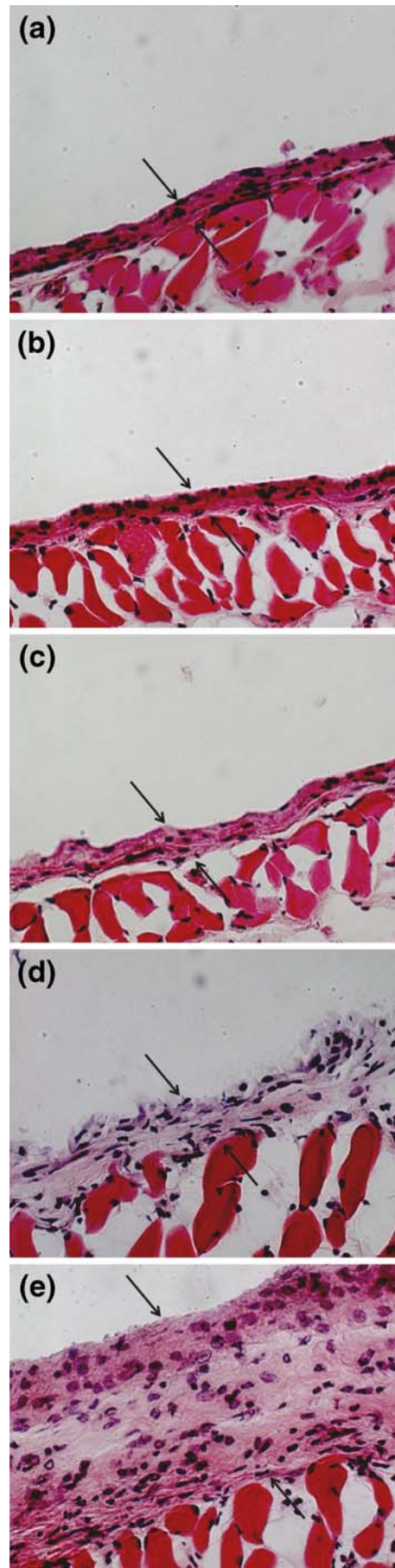


Fig. 4. Photomicrographs of representative tissue sections surrounding the implantation site with **a** DPBS (control), **b** combination system only (no VEGF), **c** single dose of VEGF, **d** VEGF-loaded microspheres in DPBS, and **e** VEGF-loaded combination system. The VEGF concentration remained constant (1 µg/animal). Original pictures were taken at 200× magnification. Arrows indicate the newly formed granulation tissue.

(50.5 mg/ml) and xylazine hydrochloride (2.9 mg/ml). The combination delivery system was subcutaneously injected into the back of mice. The mice were sacrificed 3 weeks after implantation, and tissues surrounding the injection site were retrieved. All procedures were in compliance with Hanyang University Guidelines for the care and use of laboratory animals. The tissue samples were embedded into Tissue-Tek O.C.T. compound (Sakura) in aluminum foil molds and frozen (Revco). Cryostat tissue sections (10 μm thick) were prepared using a cryo-microtome (Leica Microsystems), and stained with hematoxylin and eosin (H & E). The microscopic images were obtained using an Axioskop 40 microscope (Zeiss), and analyzed by Image-Pro Plus Software (Media Cybernetics) for quantification.

Statistical Analysis

All data are presented as the mean \pm standard deviation. Statistical analysis was performed with the 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

Combination delivery systems with various ratios of microspheres to hydrogels were prepared, and *in vitro* rhVEGF release profiles from the systems were investigated. The rhVEGF loading efficiency into the system was more than 90%, and the controlled release of rhVEGF from the combination system was achieved (Fig. 1). The release rates of rhVEGF from combination systems prepared at mixing ratios of 0, 0.5, and 1.0 (g/g, PLGA/alginate) were 6.6, 2.1, and 1.8 $\text{ng}/\text{day}^{-1}$, respectively. While rhVEGF encapsulated in alginate hydrogels only (i.e., mixing ratio=0) was almost completely released within 5 days of incubation, the sustained release was observed from combination systems. The release rate from the combination system decreased as the amount of rhVEGF-encapsulated microspheres increased in the system.

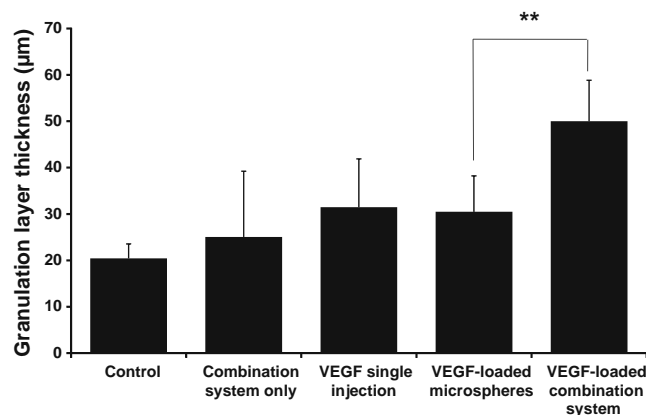


Fig. 5. The thickness of the granulation tissue layer formed around the injection site ($**P < 0.01$, $n = 6$).

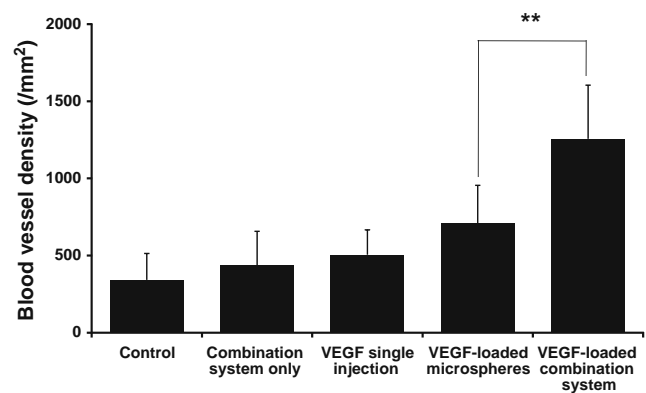


Fig. 6. The number of blood vessels formed in the granulation tissue surrounding the injection site ($**P < 0.01$, $n = 6$).

Bioactivity and Cell Proliferation

We next investigated whether the released rhVEGF remained bioactive. We tested the viability of endothelial cells cultured with EBM-2 (e.g., no growth factors in the media) after treating with either intact rhVEGF or released rhVEGF (Fig. 2). The released rhVEGF showed excellent bioactivity compared to intact rhVEGF.

In vitro proliferation of endothelial cells incubated with combination delivery systems was investigated to confirm that sustained rhVEGF release could maintain a constant therapeutic dose over time, leading to enhanced endothelial cell growth (Fig. 3). Cells cultured in EBM-2 media grew slowly due to the lack of growth factors in the media (no rhVEGF). Cell growth was not significantly affected by the presence of a combination system only (no rhVEGF). Cells treated with 100 ng rhVEGF/well showed enhanced growth, but it only lasted for a short time period and the growth rate ($0.47 \pm 0.07 \text{ day}^{-1}$) returned to baseline ($0.39 \pm 0.11 \text{ day}^{-1}$) (Table I). Interestingly, cell proliferation substantially increased when cells were cultured with the rhVEGF-releasing combination delivery system. The growth rate was remarkably high ($0.90 \pm 0.04 \text{ day}^{-1}$), compared to cells cultured with the same amount of VEGF in the media.

In Vivo Angiogenesis

We next used an animal model to test whether combination delivery systems containing rhVEGF could be useful to improve angiogenesis *in vivo*. The microsphere/hydrogel combination system at a mixing ratio of 1.0 was subcutaneously injected into the back of immunodeficient mice using a syringe (1 μg rhVEGF/mouse). Tissue sections around the injection sites were retrieved after three weeks, and the newly formed granulation tissue thickness and blood vessel density were evaluated. Granulation tissues generated near the injection site are shown in Fig. 4. The granulation tissue thickness did not change significantly, even after single injection of rhVEGF or a suspension of rhVEGF-loaded microspheres at the implantation site. The granulation tissue thickness around the combination system containing rhVEGF increased significantly. The number of blood vessels in the granulation tissue layer also increased significantly ($1,257 \pm$

348 vessels/mm²), compared with rhVEGF only (505 ± 161 vessels/mm²) or rhVEGF-containing microspheres (709 ± 246 vessels/mm²). No significant inflammatory response was observed.

DISCUSSION

This study evaluated the ability of microsphere/hydrogel combination systems containing rhVEGF, prepared by embedding PLGA microspheres into alginate gels, to provide a localized delivery system for therapeutic angiogenesis. We previously reported that the relative proportions of microspheres and hydrogels are likely to be a key factor in controlling protein release from combination delivery systems, while neither the size nor the amount of protein loaded into the microspheres significantly influences the release behavior (26). We prepared rhVEGF-loaded PLGA microspheres (8 µg rhVEGF/g PLGA) with a mean diameter of 9.7 ± 2.1 µm, which were then used for a microsphere/hydrogel combination system. We found that the initial burst of rhVEGF released from the combination systems increased as the proportion of alginate gels increased, due to rapid release of the factor from the gel. As the amount of PLGA microspheres containing rhVEGF in the combination systems increased, sustained release behavior became prevalent (Fig. 1). Localized delivery of VEGF using various delivery vehicles, including alginate gels, was previously reported (27). However, the release rate of VEGF from alginate gels was fast and most angiogenic factors were depleted within 10 days. Interestingly our combination system improved the release behavior of VEGF *in vitro* in a controlled manner for more than three weeks. In addition, released rhVEGF was bioactive and substantially promoted HUVEC proliferation *in vitro* (Figs. 2 and 3). A single dose of rhVEGF did not efficiently enhance endothelial cell growth, compared with equivalent rhVEGF loaded into a combination system. The difference was attributed to the combination delivery system protecting the factor from enzymatic degradation. Additionally, the delivery system showed no significant cytotoxicity against endothelial cells, indicating a potential use as an injectable rhVEGF delivery system *in vivo*.

We tested whether combination delivery systems containing rhVEGF could effectively enhance angiogenesis *in vivo*. Although PLGA microspheres prolonged the release behavior of rhVEGF *in vitro* (Fig. 1), insufficient blood vessels were formed *in vivo* (Fig. 4). The lack of blood vessel formation was likely due to microspheres moving from the injection site and possible phagocytotic uptake by macrophages around the injection site. The newly formed granulation tissue was thicker with significantly more blood vessels when a combination system containing rhVEGF was injected into the mice (Figs. 5 and 6). This finding clearly indicates that the localized and sustained delivery of angiogenic factors is critical for angiogenesis *in vivo*.

Using rhVEGF as an angiogenic molecule has been frequently reported to treat ischemic disease (28). VEGF genes or proteins have been widely used for therapeutic angiogenesis at ischemic sites via systemic administration (29). These applications have been limited due to imprecise gene or protein localization into ischemic sites, which may lead to unnecessary side effects (e.g., hypotension, edema)

caused by repeated administration (30). Thus, the localized delivery of angiogenic molecules using collagen (31) and PLGA (24) matrices was reported. These matrices required large doses to effectively enhance angiogenesis *in vivo*. Surprisingly, a small rhVEGF dose in our combination delivery system efficiently improved angiogenesis *in vivo*. Determining the optimum dose, systemically or locally administered, is one critical factor in regulating angiogenesis. In addition, recent studies implicated that the spatial and temporal distribution of angiogenic molecules with a minimal dosage is critical in angiogenesis (32).

CONCLUSION

We fabricated an injectable microsphere/hydrogel combination system for localized rhVEGF delivery. The sustained rhVEGF release was achieved for three weeks using this combination system. Released rhVEGF remained bioactive and enhanced endothelial cell proliferation substantially for prolonged time periods *in vitro*. Subcutaneous injection into animals significantly enhanced new blood vessel formation at a small dose. This approach may have many useful applications in avoiding unnecessary multiple administrations in treating ischemic diseases, as well as tissue engineering.

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