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RESEARCH PAPER

Co-delivery of Vascular Endothelial Growth Factor and Angiopoietin-1 Using Injectable Microsphere/Hydrogel Hybrid Systems for Therapeutic Angiogenesis

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ABSTRACT

Purpose We hypothesized that combined delivery of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) using microsphere/hydrogel hybrid systems could enhance mature vessel formation compared with administration of each factor alone.

Methods Hybrid delivery systems composed of alginate hydrogels and poly(D,L-lactic-*co*-glycolic acid) (PLGA) microspheres containing angiogenic factors were prepared. The release behavior of angiogenic factors from hybrid systems was monitored *in vitro*. The hybrid systems were injected into an ischemic rodent model, and blood vessel formation at the ischemic site was evaluated.

Results The sustained release over 4 weeks of both VEGF and Ang-1 from hybrid systems was achieved *in vitro*. Co-delivery of VEGF and Ang-1 was advantageous to retain muscle tissues and significantly induced vessel enlargement at the ischemic site, compared to mice treated with either VEGF or Ang-1 alone.

Conclusions Sustained and combined delivery of VEGF and Ang-1 significantly enhances vessel enlargement at the ischemic site, compared with sustained delivery of either factor alone. Microsphere/hydrogel hybrid systems may be a promising vehicle for delivery of multiple drugs for many therapeutic applications.

KEY WORDS angiogenesis · angiopoietin-1 · microsphere/hydrogel hybrid system · vascular endothelial growth factor

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INTRODUCTION

Ischemic vascular disease is associated with a high rate of mortality and morbidity (1), resulting in inadequate blood flow at the ischemic site (2). One potential approach for treating this disease is delivery of angiogenic factors instead of invasive surgery, which, for this condition, may carry a high risk of repeated operations (3–5). Angiogenesis is the formation of new blood vessels from pre-existing vessels, and vascular endothelial growth factor (VEGF) is known to play an important role in this process (6). As a potent mitogen, VEGF induces angiogenesis by stimulating endothelial cell proliferation, migration, and new vessel formation. Therefore, it has been tested extensively as a potential treatment for patients with ischemic limb and cardiac disease (6,7). However, it is still unclear whether VEGF alone is sufficient to promote the formation of mature vessels that can provide adequate blood flow to ischemic tissue. Moreover, excessive administration of VEGF may induce immature vessel formation, resulting in plasma leakage and tissue edema (8). Angiopoietin-1 (Ang-1) has been known to contribute to the stabilization and maturation of growing blood vessels (9) and to prevent the plasma leakage caused by excessive VEGF (10).

Although angiogenic factors have great potential as therapeutic agents, their short half-lives within the body present a practical challenge (11). Various polymer-based systems have been exploited as depots for peptides and proteins (12) to protect them from proteolysis, prolong their activity, and achieve controlled delivery of the therapeutics within the body. In particular, hydrogels have been widely used as an injectable delivery vehicle for protein drugs (13), including angiogenic factors, because various properties of hydrogels can be controlled by changing the chemical composition,

cross-linking density, and hydrophobicity (14). Alginate is a naturally occurring polymer obtained from brown algae, and has excellent biocompatibility and low toxicity (15). Additionally, simple gelation of aqueous alginate solution with divalent cations (e.g., Ca^{2+}) enables wide applicability to many drug delivery and tissue engineering systems (15). However, alginate gels typically release hydrophilic drugs rapidly. VEGF and bFGF are released from alginate gels completely within 7 days of incubation *in vitro* (16). The use of heparin-coated chitosan (17) and heparin-functionalization (18) provided means to control the release rate of VEGF from alginate hydrogels.

We previously proposed a simple but potentially valuable method to control the release behavior of protein drugs using a combination of hydrogel and hydrophobic polymer microspheres. We demonstrated that microsphere/hydrogel hybrid systems composed of poly(D,L-lactic-*co*-glycolic acid) (PLGA) microspheres and alginate hydrogels were useful for the controlled and sustained release of various proteins, including VEGF (19,20). The release behavior of proteins from hybrid systems can be regulated simply by altering the mixing ratio between microspheres and hydrogels, without the need to change the size of the microspheres (21). In addition, we found that the release rate of proteins is dependent on the porous structure of PLGA microspheres (22).

In the present study, we hypothesized that co-delivery of appropriate angiogenic factors using a microsphere/hydrogel hybrid system could enhance the maturation of newly formed blood vessels in an ischemic rodent model better than administration of a single factor. We prepared a hybrid system that releases VEGF and Ang-1 in a controlled manner, and VEGF and Ang-1 were used for promotion of neovascularization and maturation of blood vessels, respectively. Hybrid delivery systems containing angiogenic factors were injected into the ischemic site of a mouse model, and the therapeutic efficacy was evaluated.

MATERIALS AND METHODS

Materials

Sodium alginate (MW 200,000–300,000) and PLGA (RESOMER® RG 502H, MW 10,000, 0.16–0.24 dl/g) were purchased from FMC biopolymers (Drammen, Norway) and Boehringer Ingelheim (Ingelheim, Germany), respectively. Methylene chloride was purchased from J. T. Baker Chemical Company (Phillipsburg, NJ, USA). Poly(vinyl alcohol) (PVA; MW 30,000–70,000), calcium sulfate ($CaSO_4$), and ethylene diamine tetraacetic acid (EDTA) were purchased from Sigma Aldrich (St. Louis, MO, USA). Sodium hydroxide (NaOH) and hydrochloric acid (HCl) were purchased from Duksan Pure Chemicals Co. (Ansan, Korea). Dulbecco's phosphate buffered saline (DPBS) was purchased from Gibco (Grand Island, NY, USA). Recombinant human vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) were purchased from PeproTech Asia (Rehovot, Israel).

Preparation of Microspheres Containing Angiogenic Factors

PLGA microspheres containing either Ang-1 or VEGF were prepared by a water-in-oil-in-water (W/O/W) double emulsion method (23). In brief, PLGA was dissolved in methylene chloride, and each angiogenic factor was dissolved in deionized water. Both solutions were emulsified by a probe-type sonicator (Branson Digital Sonifier®; Danbury, CT, USA) for 10 s in an ice bath. The single emulsion (W/O) was poured into 4% aqueous PVA solution and emulsified again to form double emulsion (W/O/W) using a homogenizer (Ultra-Turrax® T25 basic, IKA®-Werke; Staufen, Germany) for 5 min at 6,000 rpm. The resultant double emulsion was poured into a 0.4% aqueous PVA solution and stirred at 800 rpm for 3 h to evaporate the solvent. The microspheres were washed with distilled water five times, collected, and lyophilized. The morphology of microspheres was observed by scanning electron microscopy (S-4800 UHR FE-SEM, Hitachi; Japan). To determine the protein content, microspheres were dissolved in a 1 N NaOH solution and neutralized with a 1 N HCl solution. The solution was filtered through a 0.2-μm filter (Millipore; Billerica, MA, USA) and analyzed using an ELISA assay kit (R&D; Minneapolis, MN, USA). The loading efficiency was determined by the ratio between the actual protein content in the microspheres and the amount initially added during microsphere preparation ($n=4$).

Preparation of Microsphere/Hydrogel Hybrid Systems

A microsphere/hydrogel hybrid system was prepared by ionic cross-linking of alginate solution using calcium sulfate, in which PLGA microspheres were suspended, as previously reported (21). Briefly, PLGA microspheres loaded with angiogenic factors were suspended in the DPBS (1 ml), and mixed with 3% alginate solution (2 ml) containing the factors. The solution was then cross-linked with calcium sulfate slurry (21%, w/v). All hybrid systems were prepared so that they contained equal amounts of angiogenic factors (5 μg/ml). The mass ratio of VEGF-loaded microspheres to Ang-1-encapsulated microspheres in a hybrid system was 0.5 to keep the same total concentration of angiogenic factors.

In Vitro Protein Release

The hybrid systems were cut into disks (15 mm diameter and 2 mm thick), placed in 12-well tissue culture plates containing DPBS, and incubated at 37°C under 5% CO_2

atmosphere. The supernatant was collected and the medium was changed with fresh DPBS at predetermined time intervals. The amount of each angiogenic factor present was determined using an ELISA assay kit. The release rates of Ang-1 and/or VEGF from hybrid systems were calculated by application of the power law (24).

In Vitro HUVEC Proliferation with Released VEGF and Ang-1

Human umbilical vein endothelial cells (HUVECs) were seeded onto 24-well tissue culture plates (5×10^3/well) and cultured in endothelial growth media (EGM-2) (Clonetics™, Lonza; Basel, Switzerland) for 12 h at 37°C under 5% CO_2 atmosphere. The cells were then treated with the media containing the angiogenic factors released from hybrid systems (2 ng/well), and the number of cells was counted using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The growth rate of cells was calculated from cell counts comparing the cell number at day five to the cell number at 6 h post-seeding.

Ischemic Hindlimb Model and Treatment

Mice (BALB/c nude mice, female, 4 weeks old; Orient Bio, Korea) were anesthetized by an intramuscular injection of ketamine (20 mg/kg) and Rompun® (5 mg/kg). The point above the external iliac artery and the distal point where it bifurcates into the saphenous and popliteal arteries were ligated using a 6–0 silk suture (Ailee; Busan, Korea), followed by excision of the femoral artery between them. One day after arterial dissection, the mice were randomly assigned to each experimental group ($n=7$ for each group). All the procedures were in compliance with Hanyang University guidelines for the care and use of laboratory animals. The arterial-dissected mice were treated with hybrid systems by intramuscular injection into the gracilis muscle from the medial side (injection volume = 100 μl, [angiogenic factor] = 0.5 μg/mouse). The mice treated with DPBS alone after induction of hindlimb ischemia were also used as a control. The physiological status of the ischemic limbs was observed for 4 weeks after treatment.

Histological and Immunohistochemical Analysis

Ischemic limb muscles were retrieved 28 days post-surgery. The tissue samples were embedded in an optical cutting temperature compound (O.C.T. compound, TISSUE-TEK® 4583, Sakura Finetek; Northbrook, Illinois, USA) in aluminum foil, frozen at −70°C, and then cut into 10-μm-thick sections at −20°C. Hematoxylin and eosin (H&E) and Masson's trichrome staining were performed to examine muscle degeneration, tissue inflammation, and tissue fibrosis in the ischemic regions. Fibrosis area was quantified using Image-Pro Plus Software (Media Cybernetics; Rockville, MD, USA) and expressed as a percentage of total tissue area of each image. Arterioles and capillaries in the regions were immunofluorescently stained with anti-smooth muscle (SM) α-actin (Abcam; Cambridge, MA, USA) and anti-von Willebrand Factor (vWF; Abcam; Cambridge, MA, USA), and then visualized with FITC-conjugated secondary antibodies (Jackson ImmunoResearch; West Grove, PA, USA). All the tissue sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and observed by fluorescence microscopy (TE2000-E, Nikon; Melville, NY, USA). The stained arterioles and capillaries of the tissue sections were quantified and the diameters of arterioles were determined by image analysis using Image-Pro Plus Software.

Western Blot Analysis

Tissue samples were homogenized in ice-cold lysis buffer and total protein contents of the homogenates were determined by a 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 (350 mA, 2 h), the proteins were probed with polyclonal antibodies against platelet endothelial cell adhesion molecules (PECAM, 1:200 dilution, Abcam), followed by incubation with horseradish peroxidase-conjugated anti-rabbit antibody (1:5000 dilution, Santa Cruz Biotechnology) for one hour at room temperature. The blots were developed using an enhanced chemiluminescence detection method (Amersham Bioscience), and its image was captured by a LAS-4000 luminescent image analyzer (FujiFilm).

Statistical Analysis

All data are presented as means ± standard deviation. Statistical analyses were performed using Student's *t*-test. Values of $*P<0.05$ and $**P<0.01$ were considered statistically significant.

RESULTS

Characteristics of PLGA Microspheres Loaded with Angiogenic Factors

We prepared PLGA microspheres loaded with either VEGF or Ang-1 by the double emulsion method. These microspheres have a spherical shape that was observed by scanning electron microscopy (Fig. 1a and b). Their mean diameters were 2.7 ± 0.8 μm and 3.0 ± 1.1 μm for VEGF-loaded microspheres and Ang-1 loaded microspheres, respectively. Mean diameters were determined by image analysis. The loading efficiencies of Ang-1 and VEGF in the microspheres were

88.2±6.6% and 91.6±2.5%, respectively. PLGA microspheres located within alginate gels were also observed by SEM, and no significant aggregation of the microspheres in the gels was observed (Fig. 1c).

In Vitro Release Behavior

Hybrid systems containing VEGF and Ang-1 were prepared, and the release behavior of the factors was monitored *in vitro*.

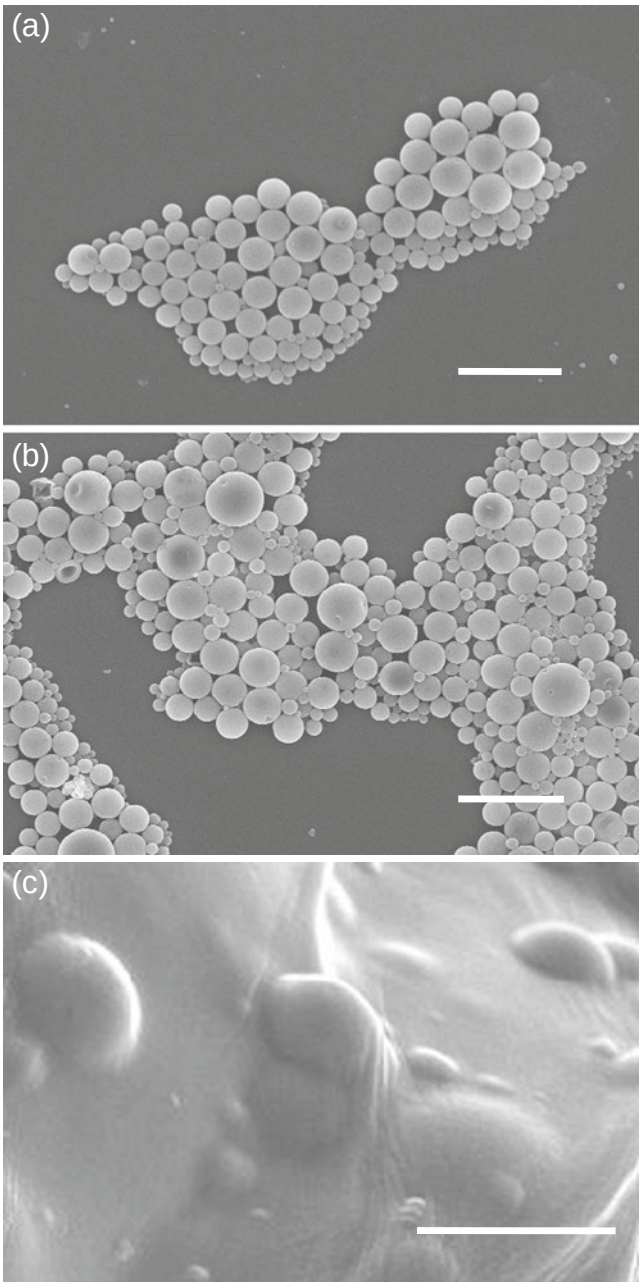


Fig. 1 Scanning electron microscope images of (**a**) VEGF-loaded PLGA microspheres, (**b**) Ang-1-loaded PLGA microspheres, and (**c**) PLGA microspheres dispersed in an alginate hydrogel in a hybrid system (scale bar = 20 μm).

Each factor alone was released from hybrid systems for 4 weeks in a sustained manner, and no substantial difference in the release behaviors was observed (Fig. 2a and b). The release rates of VEGF and Ang-1 alone from hybrid systems were 5.0±0.4%/day and 4.8±0.4%/day, respectively. Interestingly, no significant interference was observed when both VEGF (4.9±0.3%/day) and Ang-1 (4.9±0.3%/day) were loaded into hybrid systems and simultaneously released from the hybrid systems (Fig. 2c).

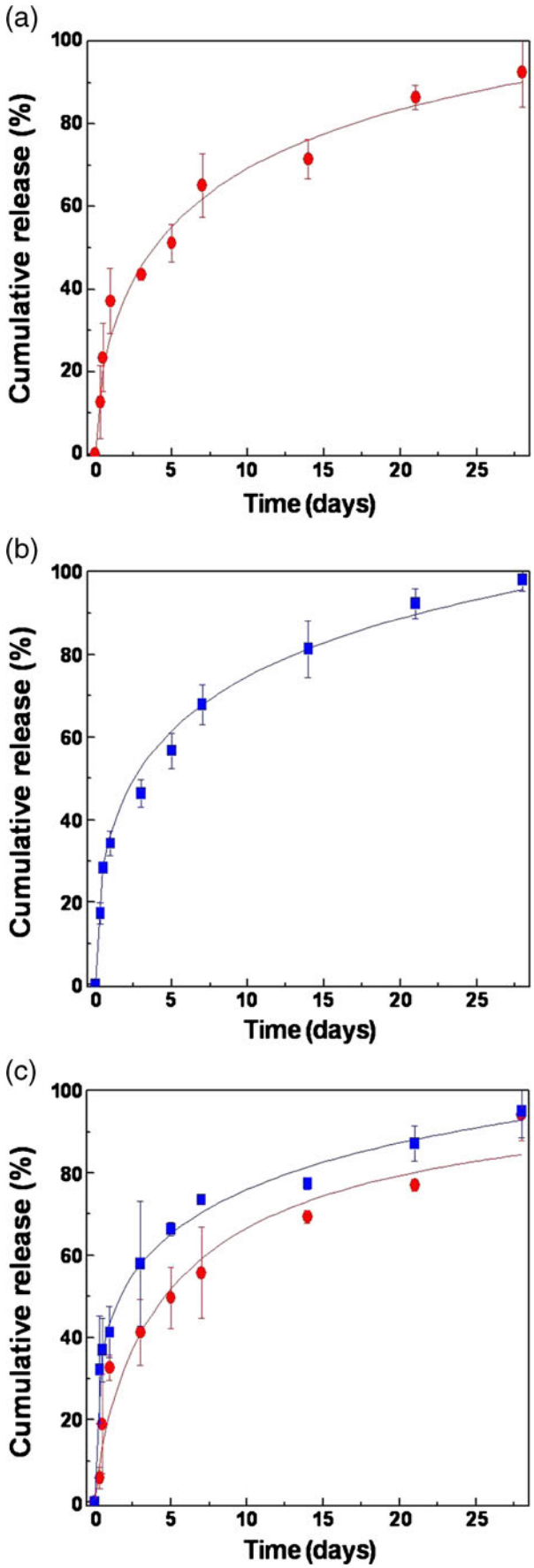
Cell Proliferation in the Presence of Factors Released from Hybrid Systems

Next, we investigated *in vitro* cell proliferation in order to test the bioactivity of released angiogenic factors. Human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 containing angiogenic factors released from hybrid systems, and their growth rate was calculated from the changes in the number of cells over time. The angiogenic factors maintained their activity after being released from the hybrid systems compared with intact ones (Fig. 3). The growth rate of cells significantly increased when the cells were cultured in media containing VEGF (0.83 ± 0.10 day^{-1} for VEGF/ANG-1=+/− and 0.93 ± 0.06 day^{-1} for VEGF/Ang-1=+/+). However, treatment with media containing only Ang-1 released from hybrid systems did not substantially influenced cell proliferation (0.54 ± 0.03 day^{-1}) compared with cells cultured in media containing VEGF. VEGF likely promotes the proliferation of HUVECs more than Ang-1.

Histological Analysis

We next tested whether co-delivery of VEGF and Ang-1 could improve therapeutic angiogenesis at the ischemic site in a rodent model. Hybrid systems were injected into the ischemic regions of mice, and tissue sections were retrieved 4 weeks after injection. The mice treated with PBS as a control (VEGF/Ang-1=−/−) showed severe foot necrosis and limb loss (Table I). However, mice treated with hybrid systems that released angiogenic factors in a sustained manner enhanced the rate of limb salvage and reduced the rate of limb loss and necrosis.

Tissue sections stained with H & E and Masson's trichrome at 28 days post-treatment also support these results. Tissue sections retrieved from a control group (VEGF/Ang-1=−/−) showed massive muscle degeneration (Fig. 4). There was prominent muscle protection from ischemic damage in groups treated with hybrid systems. Interestingly, administration of VEGF *via* a hybrid system appears to be more effective at attenuating muscle degeneration in the ischemic site than Ang-1 alone (VEGF/Ang-1=−/+). We also found hybrid systems remaining at the injection site



◀ **Fig. 2** *In vitro* release behavior of (**a**) Ang-1 alone, (**b**) VEGF alone, and (**c**) both Ang-1 (*circles*) and VEGF (*squares*) from microsphere/hydrogel hybrid systems ([protein] = 5 μg/ml, $n = 4$).

of animals after the experiment, as both gels and microspheres did not degrade during the time period that we have tested.

Immunohistochemical Analysis

We further investigated whether co-delivery of VEGF and Ang-1 using hybrid systems could effectively induce mature blood vessel formation in an ischemic mouse model. We performed immunofluorescent staining for von Willebrand factor (vWF) and smooth muscle α-actin (SM-α actin) (Fig. 5) and quantified capillary and arteriole densities (Fig. 6). Capillary density was significantly enhanced when angiogenic factors were delivered to the ischemic site using a hybrid system compared to a control group (VEGF/Ang-1 = −/−). However, capillary density was not greatly influenced by the nature of the angiogenic factor delivered to the ischemic site (Fig. 6a). Arteriole density in ischemic mice treated with hybrid systems also increased compared to the control group (Fig. 6b). Interestingly, hybrid systems containing VEGF significantly enhanced arteriole density (VEGF/Ang-1 = +/− and VEGF/Ang-1 = +/+) compared with Ang-1 release alone (VEGF/Ang-1 = −/+).

We also assessed the size and distribution of arterioles in the ischemic regions after treatment with hybrid systems. The average diameter of arterioles in the mice treated with hybrid systems containing both VEGF and Ang-1 (VEGF/Ang-1 = +/+) significantly increased compared with those treated with a single factor

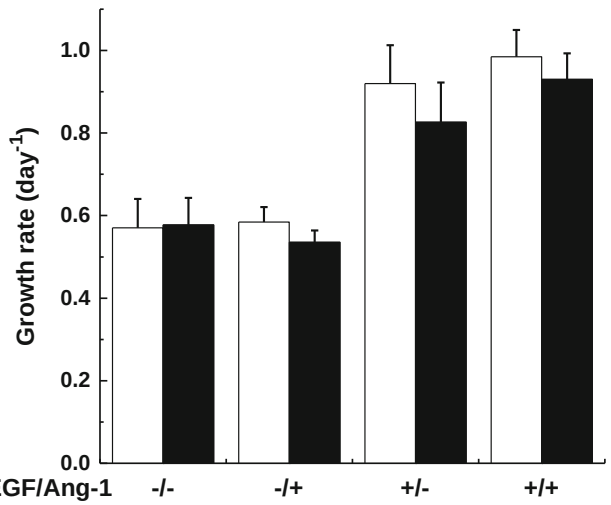


Fig. 3 Effect of angiogenic factors released from hybrid systems on *in vitro* cell proliferation. Human umbilical vein endothelial cells were cultured in endothelial growth media (EGM-2) in the presence (+) and absence (−) of either intact (*empty bars*) or released (*filled bars*) angiogenic factors ([protein] = 2 ng/well, $n = 6$), and growth rates of the cells were calculated from changes in the number of cells. As a control, cells were also cultured in EGM-2 without angiogenic factors (−/−).

Table 1 Scoring of the Ischemic Limb 4 Weeks After Treatment with Hybrid Systems Containing VEGF and/or Ang-1 ($n = 7$)

VEGF/Ang-1	−/−	−/+	+/−	+/+
Limb salvage[a]	1	5	6	6
Foot necrosis[b]	2	2	1	1
Limb loss[c]	4	0	0	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

(Fig. 7). This may indicate co-delivery of VEGF and Ang-1 is effective for vessel enlargement and maturation in ischemic tissues.

Western Blot Analysis

We tested whether hybrid systems injected into the mouse hindlimb ischemia region could induce mature, active microvessels. Prominent expression of PECAM at the ischemic site was observed *via* co-delivery of both VEGF and Ang-1 using hybrid systems, indicating functional microvessel formation (Fig. 8). Whereas, the mice treated with hybrid systems

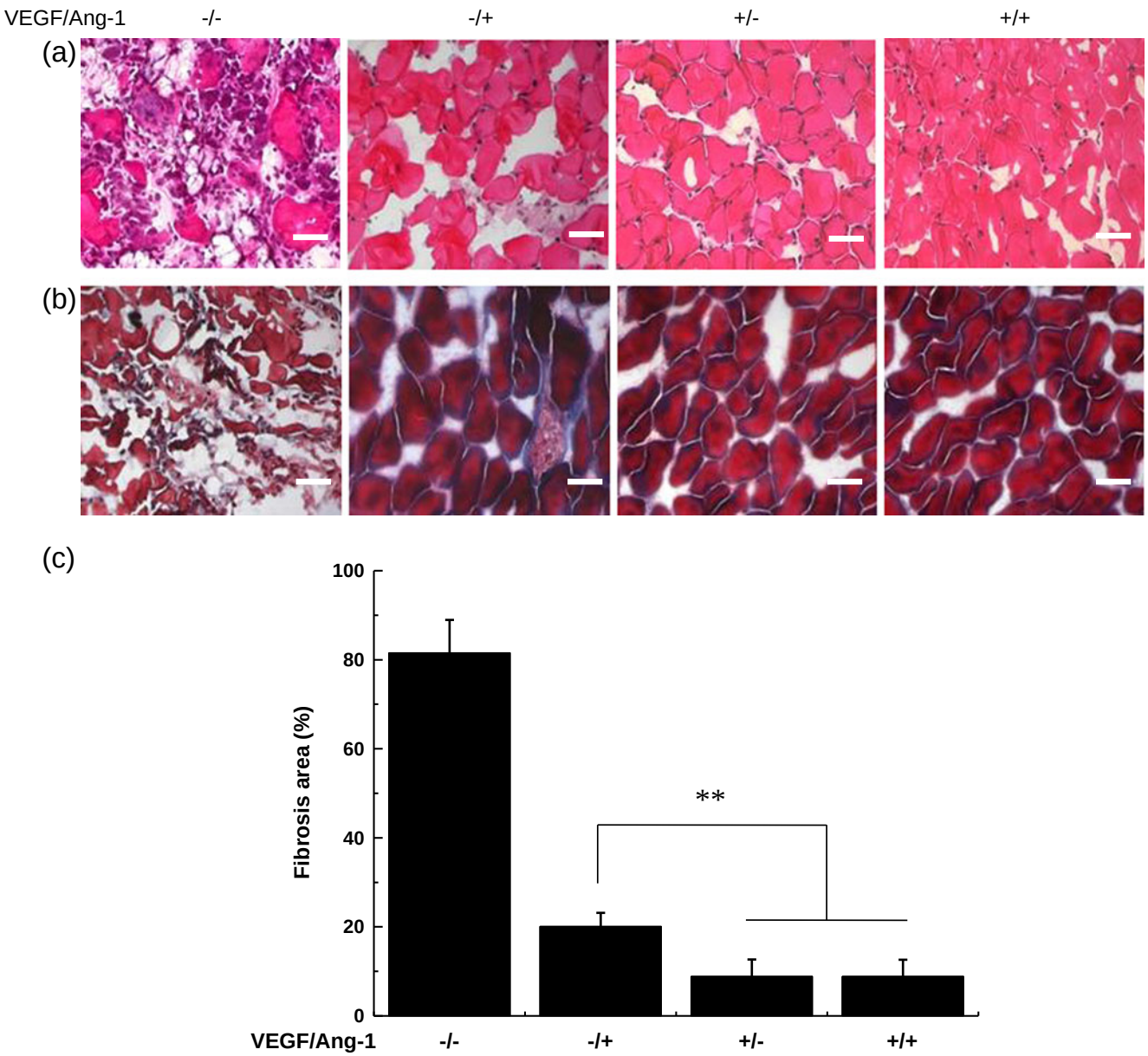


Fig. 4 Ischemic tissues treated with microsphere/hydrogel hybrid systems containing angiogenic factors. Tissue sections were retrieved from the ischemic site 28 days post-treatment and stained with (**a**) H & E and (**b**) Masson's trichrome (scale bar, 20 μm). (**c**) Quantification of fibrosis area in the ischemic regions.

containing Ang-1 alone showed reduced PECAM expression, as compared with hybrid systems containing VEGF (VEGF/Ang-1 = +/− and VEGF/Ang-1 = +/+).

DISCUSSION

New blood vessel formation is critical to patients with ischemic diseases and could be achieved by administration of angiogenic factors, as has been demonstrated in many animal studies and clinical trials (25). Neovascularization *via* branching from existing vessels could be promising for patients who are not ideal for traditional surgical methods. VEGF is one of the most potent mitogens and plays an important role in the formation of new capillaries, though it is still unclear whether VEGF is capable of inducing large vessels. Ang-1 has been known to contribute to vessel maturation and stability. Our results indicate that a combination of these angiogenic factors, locally delivered to the ischemic site of a mouse model, successfully induces large, mature blood vessel formation.

Bolus injection of therapeutic angiogenic factors into the body does not provide efficient blood vessel formation due to the short half-lives of these factors *in vivo*. Many systems allowing the sustained release of therapeutic proteins have been developed. Among them, a hybrid system composed of hydrogels and microspheres has been demonstrated to be useful for delivery of various proteins (19). The mechanical properties of alginate gels can be controlled by changes in their concentration, chain oxidation, cross-linker, and density, which are critical factors for hydrogels that are to be used as injectables. The kinetics of drug release from PLGA microspheres can be regulated by surface modification and/or introduction of porous structures to the microspheres. This variability clearly suggests that this system has potential for use as a multiple drug delivery system. Alginate gels containing PLGA microspheres are still injectable using a syringe, and no significant aggregation of microspheres in hydrogels was observed (Fig. 1c). Our findings demonstrate that the sustained release of both VEGF and Ang-1 from hybrid systems for up to 4 weeks *in vitro* can be achieved (Fig. 2c).

Angiopoietin-1 plays a key role in vascular remodeling and vessel integrity, which has often been used to induce embryonic angiogenesis, remodeling and stabilization of the neovasculature, and circulatory system development (26,27). We thus prepared hybrid delivery systems containing both Ang-1 and VEGF. Ang-1 alone did not significantly influence endothelial cell proliferation *in vitro*, and no synergistic effect on the cell proliferation was observed when both Ang-1 and VEGF were used (Fig. 3). No synergistic effect for combination of VEGF and Ang-1 on capillary formation was also observed *in vivo* (Figs. 5a and 6a). However, Ang-1 alone was effective to increase the number of capillaries in the ischemic site of mice. Interestingly, dual delivery of VEGF and Ang-1 significantly enhanced arteriole density and thickness (Figs. 5b, 6b, and 7). Prominent expression of PECAM at the ischemic site by co-delivery of both VEGF and Ang-1 indicates functional microvessel formation (Fig. 8). Ang-1 delivered in combination with VEGF promoted stable, mature blood vessel formation, which may suggest that dual angiogenic factors have a synergistic effect on therapeutic angiogenesis.

Many studies to identify the synergistic effects of multiple factors on therapeutic angiogenesis have been carried out, as neovascularization is achieved through a very complicated process (28,29). Although VEGF contributes to inducing

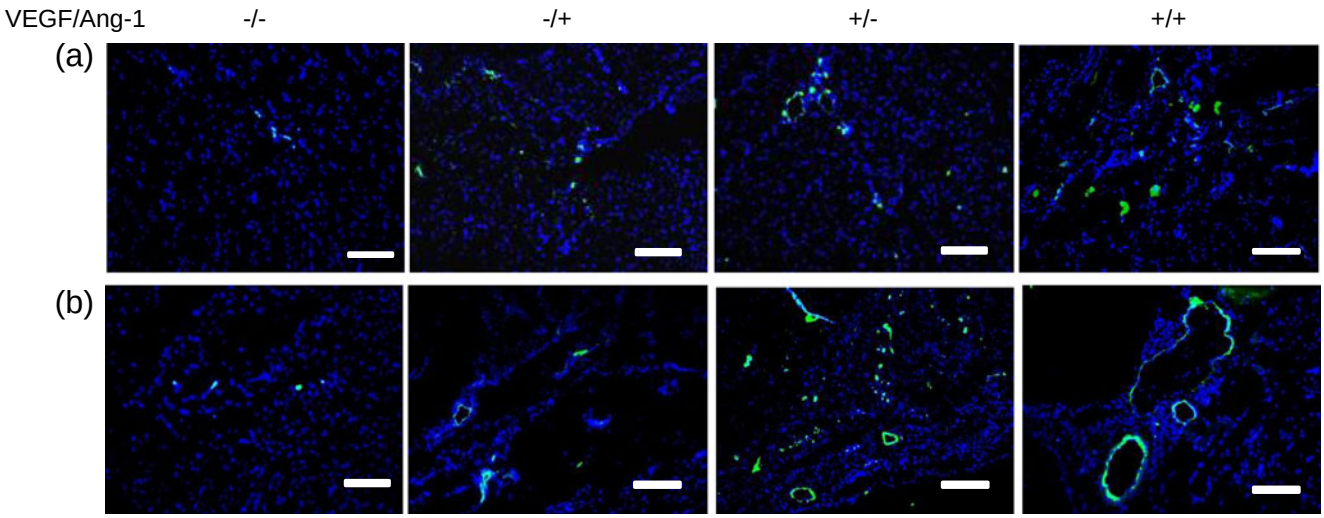


Fig. 5 Immunohistochemical staining for (**a**) vWF and (**b**) SM α-actin in ischemic tissues treated with microsphere/hydrogel hybrid systems containing angiogenic factors. *Green* represents vWF and SM α-actin, and *blue* indicates DAPI (scale bar, 40 μm).

angiogenesis at an early stage, it alone is not sufficient to develop stabilized, mature blood vessels. Many attempts to overcome this limitation through the utilization of VEGF in combination with PlGF (30), PDGF (31), and TGF-β1 (18) have been reported. However, few studies have tested whether dual delivery of Ang-1 and VEGF could promote vessel maturation compared with the delivery of a single factor (e.g., VEGF) alone, especially using microsphere/hydrogel hybrid systems. It has been reported that the genes encoding VEGF and Ang-1 significantly repressed tissue-apoptosis by promoting angiogenesis in cases of myocardial infarction in animal models (32–35). The delivery of angiogenic factors may have advantages over delivery of genes using viral vectors, including safety and controlled local concentration of the factors for a prolonged time period.

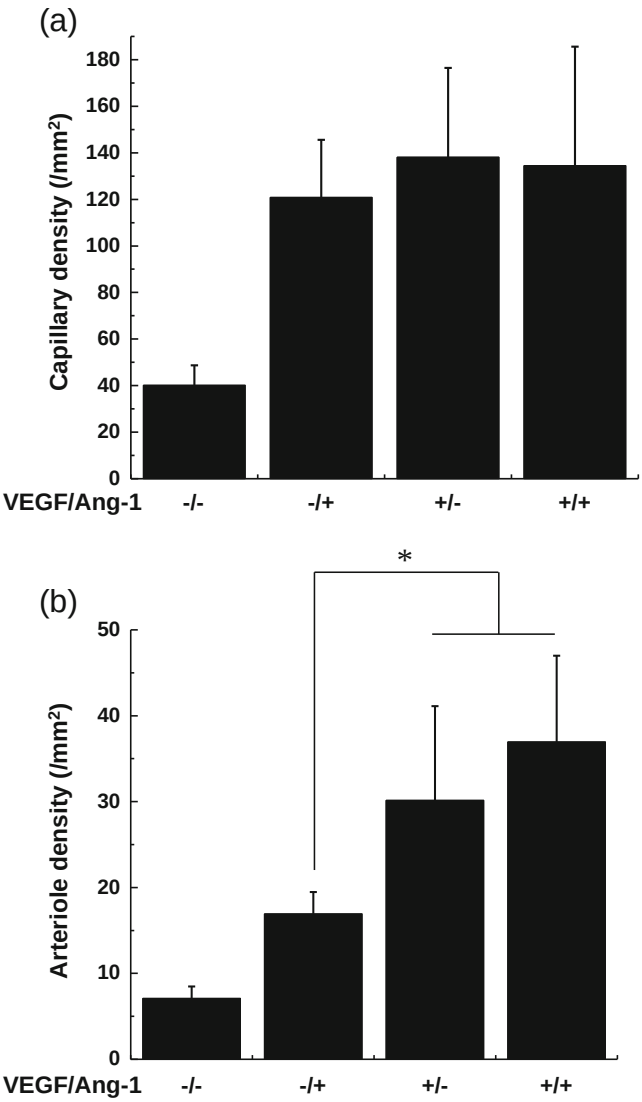


Fig. 6 Quantification of (**a**) capillary and (**b**) arteriole density in ischemic regions (*$P < 0.05$).

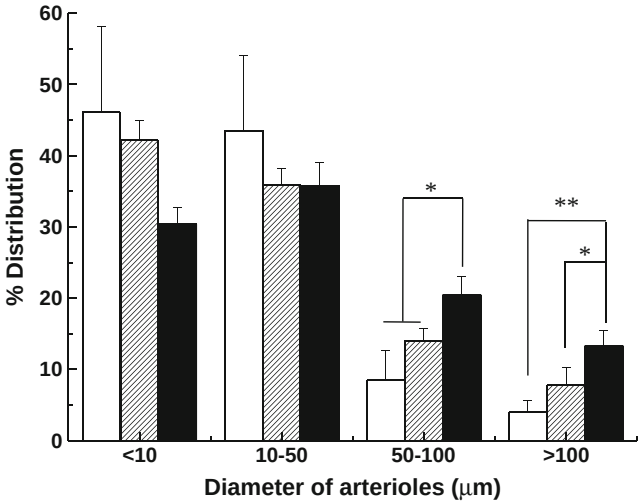


Fig. 7 Co-delivery of VEGF and Ang-1 greatly induced large blood vessel formation at the ischemic site (■). The diameters of arterioles in ischemic regions were determined by image analysis. For each condition, at least 100 blood vessels from 10 tissue sections were counted. Mice were also treated with hybrid systems releasing either Ang-1(□) or VEGF (▨) alone (*$P < 0.05$ and **$P < 0.01$).

CONCLUSION

We demonstrated that the controlled release of dual proteins can be achieved using hybrid delivery systems composed of PLGA microspheres and alginate hydrogels. Prominent muscle protection and mature vessel formation was achieved when both VEGF and Ang-1 were locally delivered to the ischemic site of a mouse model using hybrid delivery systems. This approach of controlling the release behavior of multiple drugs using hybrid delivery systems could be useful for the design and tailoring of novel drug delivery systems for many therapeutic purposes.

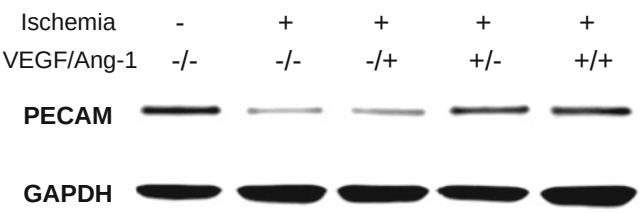


Fig. 8 Western blot analysis of ischemic tissues for PECAM. The tissue sections were retrieved from ischemic mice 28 days post-treatment with microsphere/hydrogel hybrid systems containing angiogenic factors.

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REFERENCES

1. Gaziano TA. Cardiovascular disease in the developing world and its cost-effective management. Circulation. 2005;112:3547–53.
2. Choi D, Hwang K-C, Lee K-Y, Kim Y-H. Ischemic heart diseases: current treatments and future. J Control Release. 2009;140:194–202.
3. Emanueli C, Madeddu P. Angiogenesis gene therapy to rescue ischaemic tissues: achievements and future directions. Br J Pharmacol. 2001;133:951–8.
4. Khan TA, Sellke FW, Laham RJ. Therapeutic angiogenesis: protein-based therapy for coronary artery disease. Expert Opin Pharmacol. 2003;4:219–26.
5. Losordo DW, Dimmeler S. Therapeutic angiogenesis and vasculogenesis for ischemic disease. Circulation. 2004;109:2692–7.
6. Tomanek RJ, Holifield JS, Reiter RS, Sandra A, Lin JJC. Role of VEGF family members and receptors in coronary vessel formation. Dev Dyn. 2002;225:233–40.
7. Zachary I, Mathur A, Yla-Herttuala S, Martin J. Vascular protection: a novel nonangiogenic cardiovascular role for vascular endothelial growth factor. Arterioscler Thromb Vas. 2000;20:1512–20.
8. Springer ML, Chen AS, Kraft PE, Bednarski M, Blau HM. VEGF gene delivery to muscle: potential role for vasculogenesis in adults. Mol Cell. 1998;2:549–58.
9. Chen J-X, Stinnett A. Disruption of ang-1/tie-2 signaling contributes to the impaired myocardial vascular maturation and angiogenesis in type II diabetic mice. Arterioscler Thromb Vas. 2008;28:1606–13.
10. Kim I, Moon S-O, Park SK, Chae SW, Koh GY. Angiopoietin-1 reduces VEGF-stimulated leukocyte adhesion to endothelial cells by reducing ICAM-1, VCAM-1, and E-Selectin expression. Circ Res. 2001;89:477–9.
11. Manning M, Chou D, Murphy B, Payne R, Katayama D. Stability of protein pharmaceuticals: an update. Pharm Res. 2010;27:544–75.
12. Lee KY, Yuk SH. Polymeric protein delivery systems. Prog Polym Sci. 2007;32:669–97.
13. Lee KY, Mooney DJ. Hydrogels for tissue engineering. Chem Rev. 2001;101:1869–80.
14. Tanaka Y, Gong JP, Osada Y. Novel hydrogels with excellent mechanical performance. Prog Polym Sci. 2005;30:1–9.
15. Lee KY, Mooney DJ. Alginate: properties and biomedical applications. Prog Polym Sci. 2012;37:106–26.
16. Lee KY, Peters MC, Mooney DJ. Comparison of vascular endothelial growth factor and basic fibroblast growth factor on angiogenesis in SCID mice. J Control Release. 2003;87:49–56.
17. Lee KW, Yoon JJ, Lee JH, Kim SY, Jung HJ, Kim SJ, *et al.* Sustained release of vascular endothelial growth factor from calcium-induced alginate hydrogels reinforced by heparin and chitosan. Transplant Proc. 2004;36:2464–5.
18. Freeman I, Cohen S. The influence of the sequential delivery of angiogenic factors from affinity-binding alginate scaffolds on vascularization. Biomaterials. 2009;30:2122–31.
19. Lee J, Bhang S, Park H, Kim B-S, Lee K. Active blood vessel formation in the ischemic hindlimb mouse model using a microsphere/hydrogel combination system. Pharm Res. 2010;27:767–74.
20. Lee J, Tan CY, Lee S-K, Kim Y-H, Lee KY. Controlled delivery of heat shock protein using an injectable microsphere/hydrogel combination system for the treatment of myocardial infarction. J Control Release. 2009;137:196–202.
21. Lee J, Lee KY. Injectable microsphere/hydrogel combination systems for localized protein delivery. Macromol Biosci. 2009;9:671–6.
22. Lee J, Oh YJ, Lee SK, Lee KY. Facile control of porous structures of polymer microspheres using an osmotic agent for pulmonary delivery. J Control Release. 2010;146:61–7.
23. Yang Y-Y, Chung T-S, Ping NN. Morphology, drug distribution, and in vitro release profiles of biodegradable polymeric microspheres containing protein fabricated by double-emulsion solvent extraction/evaporation method. Biomaterials. 2001;22:1231–41.
24. Siepmann J, Peppas NA. Modeling of drug release from delivery systems based on hydroxypropyl methylcellulose (HPMC). Adv Drug Deliv Rev. 2001;48:139–57.
25. Simons M, Ware JA. Therapeutic angiogenesis in cardiovascular disease. Nat Rev Drug Discov. 2003;2:863–71.
26. Peters KG, Kontos CD, Lin PC, Wong AL, Rao P, Huang L, *et al.* Functional significance of Tie2 signaling in the adult vasculature. Recent Prog Horm Res. 2004;59:51–71.
27. Saharinen P, Eklund L, Miettinen J, Wirkkala R, Anisimov A, Winderlich M, *et al.* Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. Nat Cell Biol. 2008;10:527–37.
28. Borselli C, Storrie H, Benesch-Lee F, Shvartsman D, Cezar C, Lichtman JW, *et al.* Functional muscle regeneration with combined delivery of angiogenesis and myogenesis factors. Proc Natl Acad Sci. 2010;107:3287–92.
29. Jeon O, Kang S-W, Lim H-W, Choi D, Kim D-I, Lee SH, *et al.* Synergistic effect of sustained delivery of basic fibroblast growth factor and bone marrow mononuclear cell transplantation on angiogenesis in mouse ischemic limbs. Biomaterials. 2006;27:1617–25.
30. Nagy JA, Dvorak AM, Dvorak HF. VEGF-A164/165 and PlGF: roles in angiogenesis and arteriogenesis. Trends Cardiovasc Med. 2003;13:169–75.
31. Chen R, Silva E, Yuen W, Mooney D. Spatio-temporal VEGF and PDGF delivery patterns blood vessel formation and maturation. Pharm Res. 2007;24:258–64.
32. Arsic N, Zentilin L, Zacchigna S, Santoro D, Stanta G, Salvi A, *et al.* Induction of functional neovascularization by combined VEGF and Angiopoietin-1 gene transfer using AAV vectors. Mol Ther. 2003;7:450–9.
33. Ye L, Haider HK, Jiang S, Tan RS, Ge R, Law PK, *et al.* Improved angiogenic response in pig heart following ischemic injury using human skeletal myoblast simultaneously expressing VEGF165 and angiopoietin-1. Eur J Heart Fail. 2007;9:15–22.
34. Samuel SM, Akita Y, Paul D, Thirunavukkarasu M, Zhan L, Sudhakaran PR, *et al.* Coadministration of adenoviral vascular endothelial growth factor and angiopoietin-1 enhances vascularization and reduces ventricular remodeling in the infarcted myocardium of type 1 diabetic rats. Diabetes. 2010;59:51–60.
35. Su H, Takagawa J, Huang Y, Arakawa-Hoyt J, Pons J, Grossman W, *et al.* Additive effect of AAV-mediated angiopoietin-1 and VEGF expression on the therapy of infarcted heart. Int J Cardiol. 2009;133:191–7.