Sustained Release of Erythropoietin Using Biodegradable Gelatin Hydrogel Microspheres Persistently Improves Lower Leg Ischemia

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Objectives
We hypothesized that erythropoietin (EPO)-immersed gelatin hydrogel microspheres (GHM) injected into ischemic legs might continuously release a small amount of EPO to locally stimulate angiogenesis without unfavorable systemic effects.

Background
EPO is a potent angiogenic factor, but its use for relieving ischemic organs is limited because of the untoward systemic erythrogenic effect and its short half-life in plasma.

Methods
The right femoral arteries of BALB/c mice were ligated. Recombinant human EPO (5,000 IU/kg)-immersed GHM was injected into the right hind limb muscles (n = 12); the control groups included a saline-injected group (n = 12), an EPO-injected group (n = 8), and an empty GHM-injected group (n = 8).

Results
Eight weeks later, improvement of blood perfusion to the ischemic limb was significantly augmented in the EPO-GHM group compared with any of the control groups. There was no increase in the hemoglobin level, nor was there any increase in endothelial progenitor cells. However, capillary and arteriolar densities were significantly increased in this group. Although the treatment did not affect the levels of vascular endothelial growth factor or interleukin-1 beta, it up-regulated the EPO receptor and matrix metalloproteinase-2 and activated the downstream signaling of Akt and also endothelial nitric oxide synthase in ischemic limbs, which might have been associated with the evident angiogenic and arteriogenic effects in the present system.

Conclusions
The present drug delivery system is suggested to have potential as a novel noninvasive therapy for ischemic peripheral artery disease. (J Am Coll Cardiol 2009;53:2378–88) © 2009 by the American College of Cardiology Foundation

The incidence of arteriosclerosis obliterans (ASO), the most common cause of peripheral artery disease, is estimated to be 500 to 1,000 cases per million people per year (1), and the number of patients with ASO has increased recently because of the growing prevalence of diabetes mellitus and hypertension, the aging population, and tobacco consumption. Treatment of ASO includes anticoagulants and antiplatelet drugs, percutaneous transluminal angioplasty, and bypass surgery. However, the prognosis for patients with ASO still remains poor, and amputation of the lower extremities is often required (2). Enhancement of angiogenesis and collateral arterial growth is a promising new therapeutic strategy for ASO.

The hematogenous cytokine erythropoietin (EPO) is produced by the adult kidney and is indispensable for the proliferation, survival, and differentiation of erythroid progenitor cells (3). EPO has been found to be a potent angiogenic factor. Previous studies have shown that EPO, in vitro, enhances the differentiation of endothelial cells into vascular structures (4). Furthermore, recent studies have suggested that EPO also exerts an angiogenic activity in ischemia-induced injury in the brain (5), retina (6), and heart (7). However, its use for relieving ischemic organs is limited because of an untoward erythrogenic effect (i.e., polycythemia) and its short half-life in
the plasma (i.e., 18.3 h after subcutaneous injection of 3,000 IU EPO in humans). We have developed an EPO-immersed biodegradable gelatin hydrogel sheet that gradually releases EPO and finally disappears; the sheet, when attached on the infracutaneous region, is intended to improve cardiac function and remodeling at the chronic stage (8). We hypothesized that EPO-immersed gelatin hydrogels injected into ischemic limbs might continuously release a small amount of EPO to locally stimulate angiogenesis without unfavorable systemic effects. To test this idea, in the present study we made gelatin hydrogel microspheres (GHM) (mean diameter, 30 μm) from the sheet (9), examined the effect of EPO-immersed GHM (EPO-GHM) on lower leg ischemia, and investigated the specific mechanisms of the observed effects.

Methods Preparation of EPO-GHM. The gelatin hydrogel sheet was made as previously described (8). The sheet was broken into microspheres with a mean diameter of 30 μm by a method previously reported (9). Recombinant human EPO (Chugai Pharmaceutical Co., Tokyo, Japan) incorporation into acidic and basic gelatin microspheres was carried out by allowing the freeze-dried microspheres to swell with an aqueous solution of EPO. Briefly, 15 μl of EPO solution (140 IU EPO) were mixed with 2 mg of dried glutaraldehyde-cross-linked microspheres; they were then left at 4°C for 6 h to incorporate the EPO-GHM. The microspheres were diluted with 100 μl saline just before use. Similarly, EPO-free, empty GHMs were prepared using saline without EPO. We reported that EPO was continuously released from the gelatin hydrogel sheet for at least 14 days (8), and it is known that protein release rate from gelatin hydrogels is independent of the form of gelatin hydrogels (sheet or microsphere) (9). Thus, EPO-GHM is expected to release EPO for at least 14 days.

Operative procedure and treatments. Male 9-week-old BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). This study was approved by the respective Institutional Animal Research Committees. Under sufficient anesthesia with sodium pentobarbital and local fur removal with depilatory cream, hind limb ischemia was induced by entire ligation of the right femoral artery just below the inguinal ligament. In sham-operated mice (n = 8), the suture was passed through but not tied.

After ligation, mice were randomly assigned to 4 groups: Group S (saline-treated control group), which received intramuscular injection of saline (n = 12); Group EPO, which received an intramuscular injection of EPO at a dose of 5,000 IU/kg into the hind limb (n = 8); Group GHM, which intramuscularly received a saline-immersed (empty) GHM (n = 8); and Group EPO-GHM, which intramuscularly received EPO (5,000 IU/kg)-immersed GHM (n = 12). Reagents were intramuscularly injected at 10 points in the hind limb. The dose of EPO was in a tightly controlled range of the known dosages for organ protection (10-13). Eight weeks after surgery, all of the mice were sacrificed with an overdose of pentobarbital. Muscular specimens were then subjected to immunohistochemical and molecular biological analysis.

To evaluate the role of endothelial progenitor cells (EPCs) in the present model, some of the mice were treated in the same manner, and on day 3 or at week 1 after surgery, the mice of each group (n = 6) were sacrificed. Peripheral blood was collected for fluorescence-activated cell sorter (FACS) analysis.

Laser Doppler imaging. The blood flow ratio of the ischemic limb (right)/nonischemic limb (left) was measured using a laser Doppler perfusion imager (Moor Instruments, Devon, United Kingdom). Mice were monitored by serial scanning of surface blood flow of the hind limbs before ligation, just after ligation, and on day 3 and at weeks 1, 2, 4, 6, and 8 after ligation.

Hemoglobin measurement. Hemoglobin was evaluated by HemoCue Hb 201+ (HemoCue, Angelholm, Sweden) using blood from the tail vein before and during treatment.

Mononuclear cell isolation and flow cytometry analysis. Peripheral blood mononuclear cells were isolated from each group (n = 6 each) using density gradient centrifugation with Ficoll (Immuno-Biological Laboratories, Minneapolis, Minnesota). Light density mononuclear cells were harvested and washed twice with phosphate-buffered saline. EPCs were defined by positive staining for CD34 and Flk-1 [BD Bioscience]. After incubation, erythrocytes were lysed with sodium phosphate buffered saline and analyzed with FACS Caliber (Becton Dickinson, Franklin Lakes, New Jersey). Each analysis included 100,000 events. The positive control blood sample (n = 6) was prepared from mice that were administered granulocyte colony-stimulating factor via subcutaneous injection at the dose of 100 μg/kg once daily for 5 continuous days.

Immuno histochemical analysis. Four-μm-thick paraffin-embedded sections from the lower calf muscles were incubated with primary antibodies against CD31, alpha-smooth muscle actin (1A4), muscle actin (HHF35), Ki-67, and von Willebrand factor (all from Dako Japan). An ABC kit (Vector,
Burlingame, California) was then used for immunostaining, with dianimobenzidine HCl serving as the chromogen. Quantitative assessments, including the number or area of the immunopositive cells, were made in 20 randomly chosen high-power fields (×400) using a multipurpose color image processor. For immunofluorescence, Alexa Fluor 568 and 488 (Molecular Probes, Invitrogen, Carlsbad, California) were the secondary antibodies. Nuclei were counterstained with hematoxylin or Hoechst 33342. Digital images of specimens with immunofluorescence, which were captured using a laser-confocal microscope system (LSM510, Zeiss, Oberkochen, Germany), were used for morphometric and quantitative analyses using Adobe Photoshop 7.0 software (Adobe Systems Inc., San Jose, California).

In situ nick end-labeling (TUNEL) assay was performed using an ApopTag kit (Intergene, Purchase, New York) according to the supplier’s instructions.

**Western blotting.** Lysates from hind limb muscle tissue homogenates harvested 8 weeks after surgery or cultured cells were used for Western blot analysis. Proteins were separated and transferred to membranes by standard protocols, after which they were probed with antibodies against the EPO receptor (EPOR, M-20, Santa Cruz, California). Downstream mediators of EPO receptor signaling were assessed using antibodies against Akt, the phosphorylated-Akt (p-Akt), extracellular signal-regulated protein kinase (ERK), p-ERK, and signal transducer and activator of transcription 3 (STAT3), p-STAT3, STAT5, and p-STAT5 (all from Cell Signaling, Danvers, Massachusetts). Expression and activation of endothelial nitric oxide synthase (eNOS) were assessed with antibodies against eNOS (Cell Signaling) and p-eNOS (BD Pharmingen). Matrix metalloproteinase-2 and -9 were assessed with antibodies against matrix metalloproteinase-2 (Daiichi Fine Chemical, Toyama, Japan) and matrix metalloproteinase-9 (Santa Cruz Biotechnology, Santa Cruz, California). Three to 5 right hind limb muscles from each group were subjected to blotting. The blots were visualized by means of chemiluminescence (Amersham, GE Healthcare Biosciences, Uppsala, Sweden), and the signals were quantified by densitometry. Alpha-tubulin (Santa Cruz) or β-actin (Sigma, St. Louis, Missouri) served as the loading control.

**Enzyme-linked immunosorbent assay.** Both hind limb muscles from each group (n = 3 to 5) were harvested 8 weeks after surgery and total proteins were extracted by lysing the cells in lysis buffer (50 mM Tris/HCl, pH 7.5/10 mM ethylenediaminetetraacetic acid/100 mM NaCl/0.1% Triton X-100). The levels of mouse vascular endothelial growth factor and mouse interleukin-1β were assayed with enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, Minnesota).

**In vitro cell proliferation assay.** Endothelial cells and vascular smooth muscle cells of human aorta origin (Cascade Biologics Inc., Invitrogen, Portland, Oregon) were plated into 96-well plates and cultured for 24 h, after which 5 U/ml EPO and/or 2 μmol/l wortmannin, an Akt inhibitor, was added and the cultures were continued for an additional 24 h. Then 10 ml of Cell Counting Kit-8 (Wako, Richmond, Virginia) was added to each well, and the cells were incubated for another 4 h. The accumulated formazan was then solubilized, and the absorbance at 450 nm was measured.

**Statistical analysis.** Values are shown as mean ± standard error of the mean. Statistical analysis was performed by analysis of variance followed by the Newman-Keuls multiple comparison test. For statistical analysis of blood flow ratio in Figure 1 and of EPC mobilization in Figure 4, we used a repeated-measures analysis of variance. Values of p < 0.05 were considered significant.

**Figure 1** Blood Perfusion of the Ischemic Hind Limb

(A) Laser Doppler blood perfusion. (B) Blood perfusion in ischemic hind limbs was measured before, just after, on day 3, and at weeks 1, 2, 4, 6, and 8 after right femoral artery ligation. Results are expressed as the ratio of the right (ischemic) to left (nonischemic) limb perfusion. *p < 0.05, EPO or EPO-GHM versus the other groups; #p < 0.05, EPO or EPO-GHM versus saline and GHM. EPO = erythropoietin; EPO-GHM = erythropoietin-immersed gelatin hydrogel microspheres; GHM = gelatin hydrogel microspheres.
Results

**Blood perfusion of the ischemic hind limb.** Laser Doppler imaging showed that blood flow in the ischemic hind limb was decreased equally in all groups immediately after femoral artery ligation. Although blood perfusion progressively recovered from day 3 after ischemia, improvement of blood perfusion to the ischemic limb 8 weeks after ligation was significantly augmented in Group EPO-GHM (flow ratio of ischemic to normal).
nonischemic limb, 0.81 ± 0.08), compared with any of the control groups (Fig. 1). In Group EPO, blood flow recovery increased at weeks 1, 2, and 4, but returned to the control group level thereafter.

**Neovascularization in lower ischemic hind limbs.** Immunohistochemistry for CD31 and for alpha-smooth muscle actin, an arteriole marker (14), revealed increased density of vessels and arterioles, respectively, in the ischemic limbs com-

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**Figure 3** Effects on the Proliferation or Apoptosis of Vessels in Ischemic Limbs

(A) Graphs showing the proliferating or apoptotic index of vessels at 8 weeks after ligation. *p < 0.05 versus the sham group; #p < 0.05 versus the other control groups. (B) Confocal micrographs of the ischemic limb specimen taken from the mice showing the double immunofluorescent labeling of von Willebrand factor (red fluorescence) with Ki-67 (green fluorescence). **Scale bars:** 20 μm. TUNEL = in situ nick end-labeling; vWF = von Willebrand factor; other abbreviations as in Figures 1 and 2.
pared with the sham group (Fig. 2). Vessel formation was indeed enhanced in the EPO-alone treated group at 2 weeks after surgery, when the blood flow in the ischemic limb was temporally improved, without, however, an accompanying increase of arteriolar density. In contrast, the EPO-GHM group displayed enhanced capillary and arteriolar density at 8 weeks (Fig. 2). Immunohistochemical staining for muscle actin revealed that ischemia significantly induced muscle degeneration and loss, which was not altered in any of the treatment groups, although the CD31-positive vessel to muscle cell ratio was augmented in Group EPO-GHM (Fig. 2). In nonischemic limbs, there was no significant difference in the vessel density, arteriole density, or HHF35-positive muscle area. These were similar to the levels in the sham group, indicating no influence of any treatment on nonischemic limbs.

Double immunofluorescence for von Willebrand factor and Ki-67 revealed that vessel proliferation was significantly en-

![Figure 4: EPC Mobilization in Response to Ischemia and Treatments](image)

**Figure 4** EPC Mobilization in Response to Ischemia and Treatments

(A) Evaluation of CD34+/Flk-1+ cell mobilization 7 days after treatments using FACS. (B) Graphs showing the percentages of CD34+/Flk-1+ cells, CD34+/CD133+ cells, and CD34+/Sca-1+ cells in total peripheral blood mononuclear cells (PBMC) on day 3 or 7 after surgery. *p < 0.05 versus the sham group. EPC = endothelial progenitor cell; FACS = fluorescence-activated cell sorter; G-CSF = granulocyte colony-stimulating factor; other abbreviations as in Figure 1.
hanced in ischemic limbs by EPO-GHM treatment (0.50 ± 0.09/HPF) compared with any of the control groups (Fig. 3). On the other hand, although femoral artery ligation increased TUNEL-positive vessels, there was no difference in the incidence among the ischemic groups (Fig. 3). In nonischemic limbs, there was no significant difference in Ki-67 or TUNEL positivity among the groups. Neither Ki-67–positive nor TUNEL–positive myocytes were noted in any group.

EPC mobilization in response to ischemia and treatment. Although the hemoglobin level of mice was significantly increased 2 weeks after a single injection of EPO (15.7 ± 0.20 g/dl vs. 14.4 ± 0.32 g/dl at baseline, p < 0.05), there was no significant change in the other groups, including Group EPO-GHM. Because EPO has also been reported to be a potent stimulus of EPC mobilization (15), we examined the effect of EPO on EPCs characterized by CD34+/Flk-1+, CD34+/CD133+, or CD34+/Sca-1+ expression. We found no significant difference in the number of EPCs in peripheral blood mononuclear cells between groups on days 3 or 7 after surgery (Fig. 4). Methodologic validation of our FACS analysis was confirmed by the significant increase of
EPCs in the peripheral blood of mice that received granulocyte colony-stimulating factor. It is thus suggested that EPCs are not involved in neovascularization in the present model.

Expression of EPO receptor and its downstream mediators in the ischemic hind limb. Western blot analysis showed that EPO receptor expression in ischemic limbs was significantly enhanced by EPO-GHM treatment (Fig. 5), although no difference was noted in nonischemic limbs among the groups. Western blot analysis showed that among downstream mediators of EPO receptor, ERK, STAT3, and STAT5, or their phosphorylated forms, were not related to the beneficial effects of EPO-GHM treatment, although the expression of ERK or its activation (phosphorylation) was significantly increased in ischemic limbs compared with sham (Fig. 5). On the other hand, Akt and p-Akt were markedly up-regulated in groups treated with EPO-GHM. In nonischemic legs, expression levels of...
ERK, Akt, STAT3, STAT5, and their phosphorylated forms, were similar to those of the sham-operated group, with no significant differences.

**Expression of angiogenic factors in the ischemic hind limb.** Vascular endothelial growth factor is known as a key regulator of physiological and pathological angiogenesis (16). Interleukin-1 beta is reported to play an important role in neovascularization of ischemic limbs (17). Of the cytokines tested (Fig. 6A), no significant difference in expression of vascular endothelial growth factor in the right hind limb was found with or without ligation between groups. However, expression of interleukin-1 beta was markedly up-regulated in ischemic limbs compared with sham, and it was not influenced by any treatments. In nonischemic legs, expression levels of vascular endothelial growth factor and interleukin-1 beta were similar to those of the sham-operated group, with no significant difference among the groups.

A recent study suggested that EPO also up-regulates expression of p-eNOS in carotid artery injury (18). Western blot analysis showed that p-eNOS was markedly up-regulated in groups treated with EPO-GHM, indicating activation of eNOS (Fig. 6B). Taking this finding together with up-regulation of Akt and p-Akt, it is implied that the Akt/eNOS pathway might be substantially involved in the neovascularization induced by EPO-GHM. Akt signaling is linked to activation of matrix metalloproteinase-2 and -9, which can promote angiogenesis (19). Our Western blotting revealed that matrix metalloproteinase-2, but not matrix metalloproteinase-9, was significantly up-regulated only in the ischemic legs of Group EPO-GHM mice (Fig. 6B). In nonischemic legs, there was no difference in matrix metalloproteinase-2 or -9 expression among the groups.

**In vitro effects of EPO on endothelial cells and vascular smooth muscle cells.** EPO increased cell proliferation of cultured endothelial cells, accompanying an increase in activity of Akt and eNOS, all of these were abrogated by wortmannin. In cultured vascular smooth muscle cells, EPO affected neither cell proliferation nor Akt activity (eNOS activity is absent in the basal condition) (Fig. 7).

**Discussion**

EPO-GHM and neovascularization in local ischemia. The present study demonstrated that a significant mitigation of lower leg ischemia persisted for up to 8 weeks with the treatment with EPO-GHM, implying a novel noninvasive therapy application in ischemic peripheral artery disease. Blood flow in the ischemic legs was indeed increased by a single injection of EPO, but the effect ceased at 8 weeks, in contrast to the EPO-GHM application.

In general, blood vessels form in various ways: vasculogenesis refers to the formation of blood vessels by EPCs and angiogenesis refers to sprouting from the pre-existing vessels (20). They may be subsequently stabilized by mural cells (arteriogenesis), where monocyte activation plays a major role (21). Vasculogenesis as the result of EPC activity was unlikely to be involved in neovascularization in the present model, since EPC mobilization was not altered by the applied EPO-GHM formulation (Fig. 4A), similar to a lack of erythrocytosis after treatment. Pathological findings of the present study show that EPO-GHM significantly enhanced angiogenesis in ischemic limbs without an erythrocytogenic effect. Furthermore, alpha-smooth muscle actin-positive vessels (arterioles) were also increased in the EPO-GHM group. In contrast, treatment with EPO alone induced a temporal increase in capillaries but not arterioles, suggesting that the vessels regressed because of a lack of maturation in the EPO-alone group. These findings suggest that persistence of the blood flow improvement by EPO-GHM is attributable to the formation of mature vessels (arterioles) and that long-lasting local stimulation by EPO applied with a novel delivery system is effective in ischemic legs.
is crucial for maturation and maintenance of EPO-induced new vessels.

We also observed that EPO-GHM treatment, under conditions in which significant improvement of blood perfusion was achieved, did not restore the muscle loss caused by ischemia. It is unclear at present why significant blood flow improvement did not restore or augment muscle tissue areas of the ischemic limb. However, muscle regeneration is a complex process and may consume time periods of 60 to 90 days or longer (22,23).

**Molecular mechanisms underlying the neovascularization exerted by EPO-GHM.** Our Western blot analysis showed that EPO receptor expression in ischemic limbs was significantly enhanced by EPO-GHM treatment. This is consistent with an earlier report (24), in which both EPO and hypoxia were shown to be required to up-regulate EPO receptor. In the heart and brain, EPO receptor signaling can stimulate the PI3K/Akt, Jak/STAT, and ERK/MAPK signaling pathways (7,8,10,25). EPO was recently reported to up-regulate the expression of p-eNOS in the carotid artery (18). Our findings suggest that altered signaling via Akt and eNOS, but not ERK, STAT3, or STAT5, is involved in the neovascularization induced by EPO-GHM. Our in vitro finding confirmed that EPO-mediated proliferation of ECs depends on Akt activation. Activated Akt subsequently phosphorylates and activates eNOS, leading to the production of NO (26). NO is not only a vasodilator but also is known to mediate angiogenesis (27). Another recent study using eNOS knockout mice showed that endogenous eNOS promotes angiogenesis and arteriogenesis, but has no effect in endothelial progenitor cell recruitment (28).

Kupatt et al. (29) showed that transfection of a constitutively active eNOS-mutant also enhances collateral formation (macro-arteriogenesis). These data suggest that the Akt/eNOS pathway activated by EPO is associated with cell proliferation of endothelial cells, which, in turn, induces proliferation of vascular smooth muscle cells to mature vessels. Taken together, we therefore suggest that EPO-GHM exerts not only angiogenesis but also arteriogenesis via the Akt/eNOS signaling pathway.

In addition, up-regulation of matrix metalloproteinase-2 was noted in ischemic limbs treated with EPO-GHM. Matrix metalloproteinase-2 activation, whose expression is up-regulated via Akt signaling (30), is known to promote angiogenesis through degradation of various extracellular matrix proteins (31). A recent study showed poor angiogenesis in the ischemic legs of matrix metalloproteinase-2 knockout mice (32). Although the activity remains to be confirmed because the antibodies for matrix metalloproteinase-2 used here detect only the proform, our findings suggest treatment with EPO-GHM modulates matrix metalloproteinase-2 activation in the ischemic limbs, which could promote angiogenesis.

**Discrepancies with previous studies.** Previous studies indicated that vasculogenesis occurs through circulating bone marrow-derived EPCs (33,34). EPO has also been reported to be a potent stimulus of EPC mobilization (15). In the present study, we failed to detect increased EPCs in the peripheral blood of the experimental group, suggesting little relevance of EPCs to angiogenesis by EPO-GHM. Moreover, we also noted no increase of peripheral EPCs in the other control groups. Although these observations appear to conflict with those of previous studies (15,35–37), it should be noted that there is a difference in the experimental model (mouse strain and operative procedure) between the previous studies and ours. Not unlike humans, BALB/c mice, as used in this study, show a recovery of ischemic limb perfusion of about 50% after femoral artery ligation. In contrast, the often used C57BL/6 mice are capable of entirely restoring blood flow in the ischemic limb between 2 and 4 weeks after femoral artery ligation (32,38), severely limiting the detection of long-term treatment effects. Moreover, EPC contribution to blood flow recovery in C57BL/6 mice was obtained after a more invasive procedure of complete femoral artery excision or coagulation (15,35–37) used, which might release more proangiogenic signals to the bone marrow, in turn mobilizing EPCs more efficiently. Furthermore, administration protocols of EPO were different: a single injection of 5,000 U/kg EPO in our study versus multiple-time injection of 1,000 IU/kg EPO 6 times in C57BL/6 mice (15) or 5,000 IU EPO 8 times in humans (39). Frequent stimulation by EPO as well as the total amount of EPO may be important for inducing EPCs into peripheral blood.

**Clinical implications.** The present study reports promising results of a new method of therapeutic angiogenesis for treatment of limb ischemia with a slow-release system of EPO using biodegradable gelatin hydrogels. At present, both EPO and gelatin hydrogels are already in use in the clinical setting, with safety clearly established, so the unfavorable systemic effect of EPO is avoidable with this method. We believe that this therapy is safer, less invasive, and more promising compared with previous therapeutic angiogenesis and is readily applicable to patients suffering from peripheral artery disease.

**Acknowledgments**

The authors thank Drs. Toshihiro Kushibiki, Takahiro Okasora, Toshihiro Ogawa, and Norio Doi (Kyoto University) for help in making gelatin hydrogels, and Akiko Tsujimoto and Kazuko Goto (Gifu University) for technical assistance.

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Key Words: angiogenesis • erythropoietin • ischemia • vascular disease.