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ORIGINAL ARTICLE

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Enhanced angiogenesis by gelatin hydrogels incorporating basic fibroblast growth factor in rabbit model of hind limb ischemia

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Abstract Recently we have developed new sustained release system of basic fibroblast growth factor (bFGF) using gelatin hydrogel as a carrier. Using this system, we examined the effect of topical sustained release of bFGF on angiogenesis and tissue blood perfusion in a rabbit model of hind limb ischemia. Thirty-two rabbits underwent excision of right femoral artery under general anesthesia. Two weeks later the rabbits were randomized into four groups (n = 8 each): no treatment, intramuscular injection of gelatin hydrogel alone, and intramuscular injection of gelatin hydrogel incorporating 30 μg and 100 μg of bFGF. Four weeks after each treatment, selective angiography, tissue blood flowmetry using laser Doppler perfusion imaging, and histological examination of thigh muscle were performed. In groups treated with bFGF incorporating gelatin hydrogel, tissue blood flow, number of arterioles, and vascular density were significantly increased in a dose-dependent manner 4 weeks after the treatment. Serum concentrations of bFGF and vascular endothelial growth factor were not elevated 4 weeks after the treatment. In conclusion, sustained release of bFGF using gelatin hydrogel augmented angiogenesis and improved tissue blood flow after excision of the femoral artery.

Key words Growth factor · Limb ischemia · Angiogenesis · Drug delivery

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Introduction

Despite advances in techniques of surgical and catheter interventions, major limitations still exist in the treatment of patients with severe peripheral vascular disease.[1] Recently, therapeutic angiogenesis using some growth factors has entered the spotlight both in experimental and clinical studies for the treatment of vascular obstructive diseases.[2–4] Among these growth factors, basic fibroblast growth factor (bFGF) seems to be suitable for therapeutic angiogenesis, because it is one of the potent mitogens regulating proteins that induce the proliferation of epithelial and mesenchymal cells and promoting the growth and regeneration of organs and tissue in vivo. We have developed a new sustained release system of bFGF using gelatin hydrogel,[5,6] and have reported that application of the gelatin hydrogel incorporated with bFGF markedly accelerated the healing of devascularized sternum in vivo.[7–9] Recently, we have found that intramyocardial injection of bFGF-incorporating gelatin hydrogel improved angiogenesis and ventricular function after myocardial infarction in the rat.[10] In the present study, we examined the efficacy of topical use of gelatin hydrogel incorporating bFGF in a rabbit model of hind limb ischemia.

Materials and methods

Preparation of gelatin hydrogels incorporating bFGF

Gelatin with an isoelectric point of 4.9 was isolated from bovine bone collagen by an alkaline process using $Ca(OH)_2$ (Nitta Gelatin, Osaka, Japan). Human recombinant bFGF with an isoelectric point of 9.6 was supplied by Kaken Pharmaceutical (Tokyo, Japan). Gelatin hydrogels were made as previously described.[6] Gelatin hydrogels were impregnated with an aqueous solution containing 30 or 100 μg of bFGF, to obtain gelatin hydrogels that incorporated bFGF (hydrogel bFGF). All experimental processes were conducted under sterile conditions.

Animal experiments

Thirty-two Japanese white rabbits weighing between 3.0 and 3.5 kg were used. Each rabbit was sedated with intravenous sodium pentobarbital (20 mg/kg), and allowed to breathe room air. The body temperature was maintained at 37°C with a warming pad. After local anesthesia with subcutaneous lidocaine hydrochloride (3 mg/kg), a longitudinal incision was made on the right medial thigh region from groin to knee. Then the entire right femoral artery was dissected. After all the branches were ligated as well as the popliteal and saphenous arteries, the femoral artery was ligated at its origin from the external iliac artery and was completely excised. Two weeks later, each animal underwent angiography and laser Doppler blood flowmetry of hind limb as described below.

These rabbits were then divided into four groups ($n = 8$ each) according to the additional treatment: no treatment (group A), injection of gelatin hydrogel alone into medial thigh muscle (group B), and intramuscular injection of 30 μg and 100 μg of hydrogel bFGF (groups C and D, respectively). Four weeks after the treatment, a second angiography and laser Doppler blood flowmetry were performed. Then each rabbit was killed by an overdose of sodium pentobarbital, and the medial thigh muscles of the ischemic limb were harvested and were subjected to histological examination.

All animals used in this study received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication 85-23, revised 1985), and all of the animal experiments were performed according to the institutional guidelines on animal experiments of Kyoto University.

Angiography

All animals were subjected to abdominal aortography before and after treatment. Each animal was sedated intravenously with sodium pentobarbital and an incision was made in the neck after local anesthesia using lidocaine hydrochloride. The left carotid artery was exposed, and a 5-F catheter was cannulated with its tip positioned just above the aortic bifurcation in the lower abdominal aorta. Angiography was performed by manual injection within 2 s with 2 ml of nonionic contrast media (Iomeprol, Eisai, Tokyo, Japan).

Measurement of tissue blood flow by laser Doppler imaging

Blood flow of hind limbs was measured by laser Doppler perfusion imaging (LDPI) as described otherwise.[11] In brief, the LDPI system (Moor Instruments, Devon, UK) uses a 12-mW helium-neon laser beam that sequentially scans a 5 × 5-cm surface area. During the scanning procedure, the LDPI system detects frequency shifts of the laser caused by blood cells moving through the vasculature according to the Doppler principle. The LDPI system transforms them into voltage variations that represent blood perfusion of the scanned area. This perfusion signal is split into 14 different intervals, and each is displayed as a separate color. In the present study, the mean value of perfusion signal was calculated for each scanned area. In each animal, improvement of the perfusion signal obtained 4 weeks after treatment was expressed as a percentage of the signal before treatment.

Histological assessment of angiogenesis

For conventional histological examination, the medial thigh muscles of the ischemic limbs were excised in each animal after death. After being fixed in PBS-buffered 10 wt% formaldehyde solution for 4 days, each sample was embedded in paraffin, cut into 10-μm thick slices, and stained with hematoxylin – eosin.

For immunohistochemical staining, the lower limb of each rabbit was perfusion-fixed with 4% paraformaldehyde after sacrifice. Then the medial thigh muscles of the ischemic limbs were excised and embedded in optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan) and frozen at –80°C. Cryostat sections (5 μm thick) of the tissues were stained with monoclonal antibody for von Willebrand factor (vWF) (DAKO Japan, Kyoto). Five slices were randomly selected from each sample, and the numbers of arterioles (external diameter between 25 and 100 μm) and capillaries (external diameter less than 25 μm) were counted in each slice by two pathologists blinded to the treatment.

Measurement of bFGF and VEGF

Blood samples for measurement of serum concentration of bFGF and vascular endothelial growth factor (VEGF) were obtained from the carotid artery at the time of angiography. Serum concentrations of bFGF and that of VEGF were measured by an enzyme-linked immunosorbent assay with murine monoclonal antibodies specific for bFGF and VEGF (Quantikine, R&D systems, Minneapolis, MN, USA), respectively.

Statistics

Experimental results are expressed as mean ± SD. Differences between four experimental groups were analyzed by analysis of variance followed by Scheffé's post hoc test. Comparisons between two groups were made using upparied two-tailed Student's *t*-test or chi-square test. Statistical significance was defined as a P value of less than 0.05.

Results

Tissue blood flow in hind limb

Results of tissue blood flow evaluated by LDPI are shown in Fig. 1 (left panel). In group A and group B, tissue blood flow 4 weeks after treatment was not significantly different

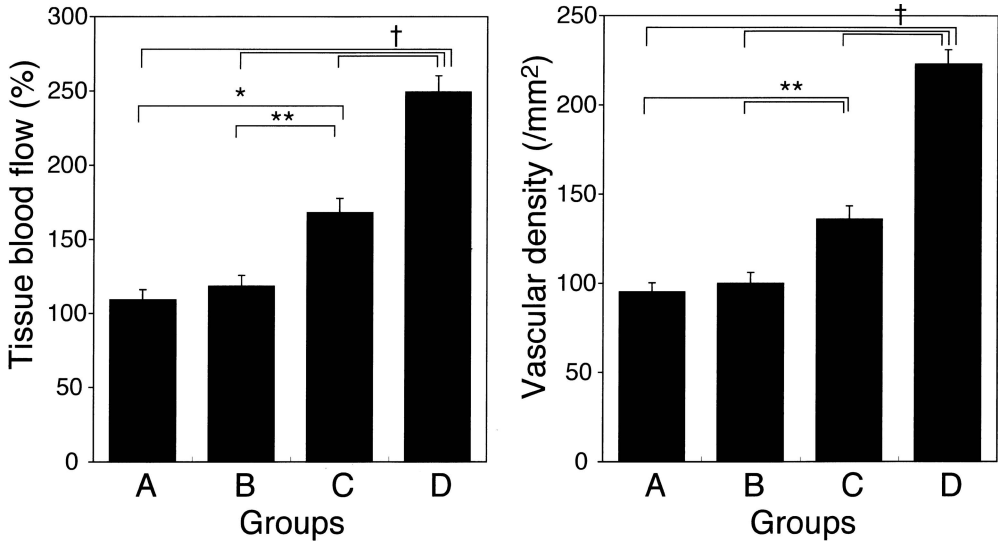

Fig. 1. Tissue blood flow (**left**) and vascular density (**right**) in each group 4 weeks after the treatment. Injection of hydrogel basic fibroblast growth factor (bFGF) (groups C and D) significantly augmented tissue blood flow and vascular density in a dose-dependent manner. * $P < 0.001$, ** $P < 0.01$, † $P < 0.0001$

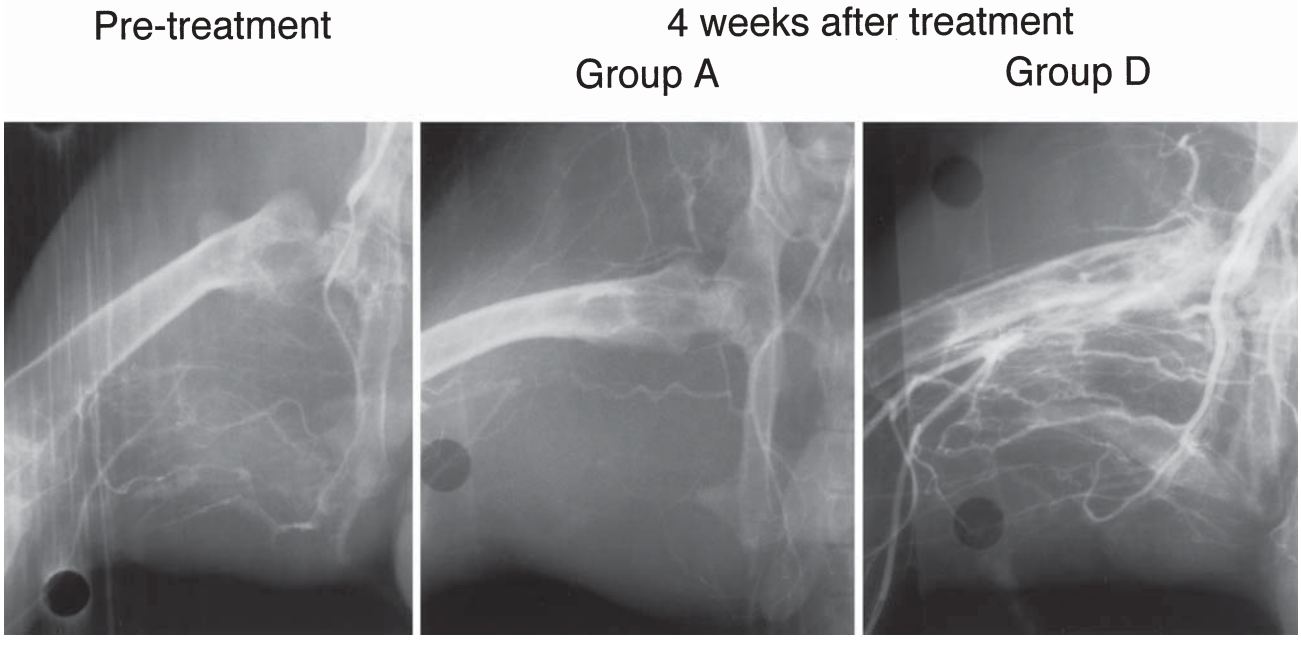

Fig. 2. Representative angiograms of right hind limb. In the group treated with 100μg of bFGF (group D), collateral vessel formation was markedly increased compared to pretreatment and group A (no treatment)

from pretreatment value (109.1% ± 6.9% and 118.5% ± 7.2% of pretreatment value, respectively). In contrast, in groups treated with hydrogel bFGF, tissue blood flow significantly increased according to the dose of bFGF (168.1% ± 9.5% in group C and 249.4% ± 10.8% in group D).

Angiographic assessment

Representative angiograms of groups A and D are shown in Fig. 2. In the animals without any treatment (group A), collateral vessel formation was about the same as that before treatment (2 weeks after arterial excision). In contrast, in the animals that were treated with 100μg of hydrogel bFGF (group D), collateral vessel formation was markedly augmented 4 weeks later.

Histological assessment of angiogenesis in ischemic hind limb

Figure 3 shows representative microscopic views of medial thigh muscle in group A and group D. Arterioles (indicated by black arrows in H&E-stained slices) were scarcely observed between muscle tissue in group A, while more arterioles were observed in group D. As shown in slices stained with vWF antibody (Fig. 3b), a significant increase of vascular density was observed in groups treated with hydrogel bFGF. Vascular density in groups A–D is 95.1 ± 5.1, 100.0 ±

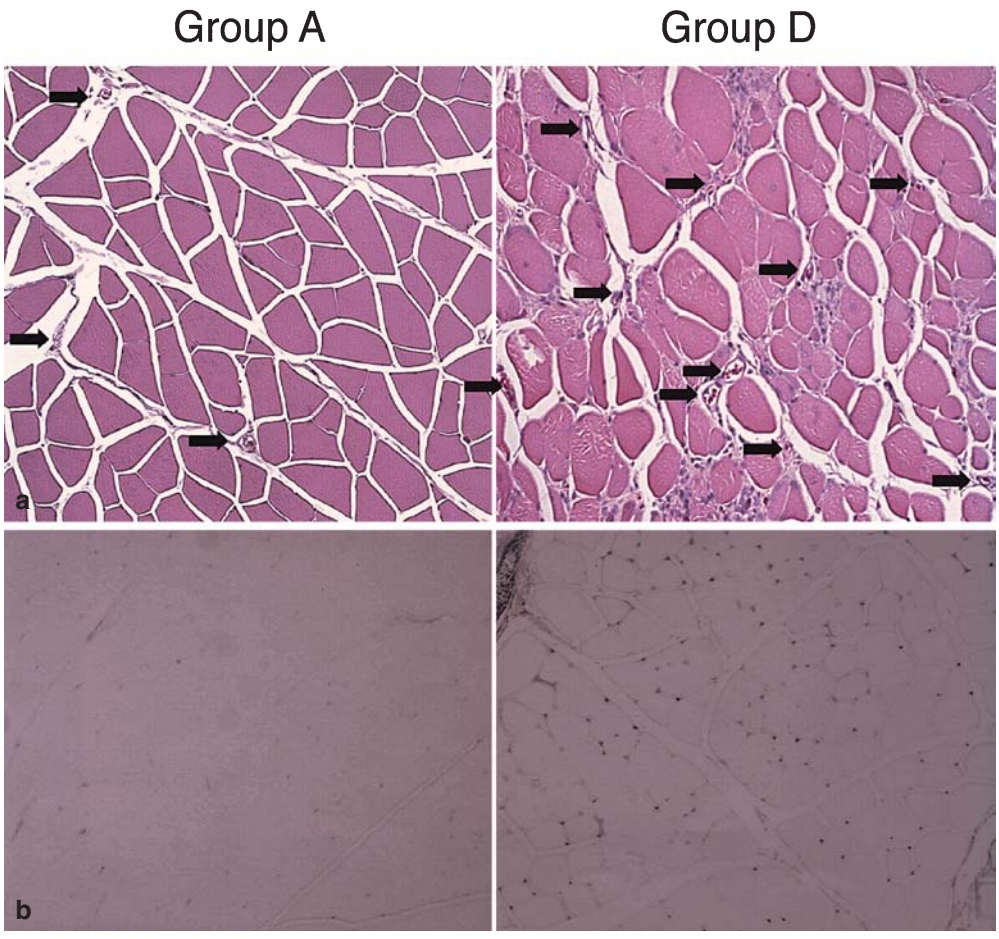

Fig. 3a,b. Representative microscopic photos of right medial thigh muscle 4 weeks after the treatment. Upper slices **(a)** were stained by H&E, lower slices **(b)** were stained by von Willebrand factor antibody (×20). *Arrows* in H&E staining indicate arterioles. As shown in lower slices **(b)**, the number of vessels are greater in group D (treated with 100 μg of bFGF) than in group A

6.1, 136 ± 7.4, and 223 ± 8.0/mm^2, respectively (Fig. 1, right panel).

Serum concentrations of bFGF and VEGF

Serum concentrations of bFGF and VEGF before treatment were 17.9 ± 2.4 and 14.1 ± 0.8 pg/ml, respectively. Four weeks after each treatment, serum concentrations of bFGF in groups A–D were 15.1 ± 7.9, 18.1 ± 7.9, 12.8 ± 12.3, and 14.7 ± 6.4 pg/ml, and those of VEGF were 12.7 ± 6.0, 13.9 ± 1.3, 14.1 ± 2.6, and 12.9 ± 3.4 pg/ml, respectively. No significant difference was observed either between groups or between before and after the treatment.

Discussion

In the present study, we have demonstrated that single intramuscular injection of gelatin hydrogel incorporated with bFGF significantly promotes the improvement of tissue blood flow after excision of femoral artery in rabbit. In animals treated with hydrogel bFGF, vascular density increased significantly 4 weeks later in dose-dependent manner and improvement of tissue blood flow was obtained in parallel with the vascular density. Basic FGF has a potent angiogenic effect and has been considered to be suitable for terapeutic angiogenesis. Although the biological half-time of bFGF in free form is short, it has been reported that bFGF enhanced growth of collateral vessels in vivo by multiple injection[12] or sustained release using heparin-alginate microspheres.[13,14] Our result is comparable with those reports. Considering that our method necessitates only a single intramuscular injection, it is more feasible than multiple injection of free bFGF from the clinical point of view.

Thus far, a sustained-release system using heparin-alginate microspheres has been applied in some experiments.[13,14] In this system, bFGF carrier is an alginate microsphere containing heparin-Sepharose beads. The release of heparin-bound bFGF is achieved through cleavage of heparin by heparinase, which is suppressed by incorporation into the calcium-cross-linked alginate microsphere. Since the alginate is poorly biodegradable and its erosion depends on natural leaching out of the calcium, it may be difficult to control the bFGF release. In addition, the alginate microsphere as well as nonbioldegradable Sepharose beads would remain in the body after bFGF is fully released. In contrast, in our gelatin hydrogel system, bFGF is released as a result of degradation of gelatin, which can be controlled by changing the water content of gelatin hydrogels.[15] Moreover, the gelatin hydrogel is completely degraded in the body, which would not cause inflammatory and pharmacological response in vivo. In the present study,

leukocyte counts and C-reactive protein concentrations in peripheral blood before and after treatment did not show significant differences between each treatment group (data not shown).

Another way to manage the shortness of half-time is gene transfer of growth factors. Compared to our method, gene transfer has some disadvantages. Gene transfer necessitates use of vectors or plasmids, whose expression could not be fully controllable after administration in vivo. Furthermore, gene transfer has a poorly and incompletely understood mechanism of action. In contrast, our method is local protein delivery, in which it is much easier to regulate duration and potency of action of growth factors. In the present situation, we consider that delivery is much closer to practical use than gene therapy.[16]

Recently, transplantation of autologous bone marrow cells has been reported to improve limb ischemia by formation of collateral vessels, and some clinical pilot studies have been done.[17] Further clinical studies are necessary to find out which method is more effective in the treatment of severe limb ischemia, b-FGF slow release or bone marrow cell transplantation. One of the advantages of our method in the clinical situation is its independence from invasive preparations before the therapy. To collect sufficient bone marrow cells, it is necessary to puncture bone marrow and aspirate up to 500 ml of marrow under general anesthesia. Pretreatment with granulocyte-colony stimulating factor could reduce the requisite volume of marrow cells, but it might cause deleterious vascular events because of leukocytosis or hypercoagulability.

One of the major possible problems of therapeutic angiogenesis using growth factors is systemic side effects. Systemic administration of bFGF could induce angiogenesis in undesired organs or development of neoplasm. In the present study, serum levels of bFGF and VEGF did not rise after the treatment, suggesting that systemic effects were negligible by our method. Nevertheless, further study including histological examination of major systemic organs might be necessary to confirm the safety of our method.

In conclusion, the present study shows the promising results of a new method of therapeutic angiogenesis for the treatment of limb ischemia using a slow-release system of bFGF. We believe that this therapy helps prevent limb amputation, and we have started a clinical trial recently.

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