Combined Treatment of Sustained-Release Basic Fibroblast Growth Factor and Sarpogrelate Enhances Collateral Blood Flow Effectively in Rabbit Hindlimb Ischemia

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Background The effectiveness of sustained-release basic fibroblast growth factor (bFGF) in potentiating arteriogenesis and angiogenesis was evaluated, as well as determining whether chronic oral administration of sarpogrelate, a serotonin blocker, would further increase collateral blood flow in the rabbit hindlimb following surgical induction of ischemia by femoral artery extraction.

Methods and Results Two weeks after femoral artery removal, the rabbits were assigned to 1 of 4 experimental groups and treated for 4 weeks: group A, no treatment; group B, supplemented with diet containing sarpogrelate; group C, single intramuscular injection of sustained-release form of bFGF microspheres; group D: combined treatment with sustained-release bFGF and sarpogrelate. Endpoint measurements performed at 6 weeks found that the ischemic hindlimb blood flow was significantly improved in the rabbits that received sustained-release bFGF, with a further significant improvement in those with the additional administration of sarpogrelate. Angiographic assessment revealed augmented density of collateral vessels in the medial thigh region in the rabbits given the combined treatment.

Conclusions The findings demonstrate that sustained-release bFGF stimulated the development of collateral vessels, and additional administration of sarpogrelate produced a further improvement in hindlimb blood flow in the rabbit hindlimb ischemia model. (*Circ J* 2006; 70: 1190–1194)

Key Words: Basic FGF; Collateral circulation; Hindlimb ischemia; Sarpogrelate; Serotonin blocker

Acute or chronic interruption in blood flow in peripheral arterial disease (PAD) results in inadequate tissue perfusion, cellular damage and loss of function. With the current rise in the geriatric population worldwide, the prevalence of PAD is now increasing. The best therapeutic strategies are interventional or surgical procedures to either reopen or to bypass the occlusion to reestablish blood flow to the ischemic region. One alternative approach to treating PAD is to promote the inherent ability to develop collateral vessels that improve and maintain blood flow to the ischemic area.

Augmentation of collateral blood flow is achieved by the growth and dilation of the preexisting collateral vessels. Collateral growth encompasses the proliferation of capillaries in the ischemic area (angiogenesis) and the maturation of preexisting collateral vessels (arteriogenesis), with the latter being more important in terms of increasing the collateral blood flow.^{1–5}

Therapeutic angiogenesis with growth factors has been introduced for the treatment of PAD^{6,7} but although local delivery of basic fibroblast growth factor (bFGF) augmented collateral circulation in a rabbit hindlimb ischemia model⁸ the limitation of the use of recombinant bFGF lies in its low accumulation in the ischemic tissue and its rapid inactivation in vivo. Gelatin hydrogel microspheres incorporating bFGF have been developed for sustained-release⁹ and we recently demonstrated the effectiveness of a sustained-release bFGF system in various pathological conditions, including hindlimb ischemia.^{10–12}

The dilation of collateral vessels is achieved by administration of a serotonin blocker, sarpogrelate,^{13,14} because newly developed collateral vessels are constricted by platelet activation or endothelial dysfunction.^{15,16}

Thus, the purpose of the present study was to evaluate the effectiveness of sustained-release bFGF for potentiating arteriogenesis and angiogenesis, and to determine whether chronic oral administration of sarpogrelate further increases the collateral blood flow in a rabbit hindlimb ischemia model.

Methods

Animal Model

Forty-four adult Japanese White rabbits (male, 3–4 kg) were used for the completion of this study. All procedures related to the use of animals were approved by the Institu-

(Received April 10, 2006; revised manuscript received June 12, 2006; accepted June 30, 2006)

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tional Animal Care and Use Committee at Kyoto University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The initial surgery to induce hindlimb ischemia has been described previously.¹² Briefly, the rabbits were anesthetized with sodium pentobarbital (40 mg/kg, im). Under sterile surgical conditions, a longitudinal incision was made in the medial thigh of one hindlimb, extending from the inguinal ligament to a point just proximal to the patella, to expose and isolate the external iliac artery, which was ligated twice and sectioned between the ligatures. The femoral artery was completely excised from its proximal origin to the point where it bifurcates into the saphenous and popliteal arteries. Blood flow to the hindlimb after excision of the femoral artery is dependent upon flow through the internal iliac artery. The incision was closed in 3 layers with 3.0 nylon filament. All rabbits were closely monitored by veterinary staff and received an antibiotic (cefazolin, 20 mg/kg, s.c.) for 3 days following surgery.

Preparation of Gelatin Hydrogel Microspheres Incorporating bFGF

Gelatin hydrogel microspheres incorporating bFGF were prepared as follows. Gelatin with an isoelectric point (IEP) of 4.9 was isolated from bovine bone collagen by an alkaline process using $\text{Ca}(\text{OH})_2$ (Nitta Gelatin, Osaka, Japan). Gelatin microspheres were prepared through glutaraldehyde cross-linking of gelatin in aqueous solution dispersed in an oil phase. A mixture of the gelatin aqueous solution (10 wt%, 0.2 ml) and olive oil (5 ml) was preheated to 40°C, followed by agitation for 1 min. The emulsion prepared was cooled down in crushed ice to allow the formation of natural gelatin for the gelatin aqueous solution. Acetone was then added to the emulsion and stirring was continued for 1 h at 4°C. The resulting microspheres were washed 3 times with acetone (4°C) and recovered by centrifugation at 4,500 g and 4°C for 5 min. The non-cross-linked gelatin microspheres (20 mg) were placed in 20 ml of 0.1 wt% Tween-80 aqueous solution containing 0.13 wt% of glutaraldehyde, and stirred at 4°C for 24 h to facilitate cross-linking. Following collection by centrifugation (4,500 g, 4°C, 5 min), the microspheres were agitated in 20 ml of 10 mmol/L aqueous glycine solution at 37°C for 1 h to block the residual aldehyde groups on unreacted glutaraldehyde. The resulting microspheres were finally washed 3 times with double-distilled water by centrifugation and freeze-dried. The microsphere diameter was measured for at least 100 microspheres under a light microscope to calculate their volume. The average diameter of the microspheres was 10 µm.

Human recombinant bFGF with an IEP of 9.6 was supplied by Kaken Pharmaceutical, Tokyo, Japan and incorporated into the gelatin microspheres by dropping 5 mg/ml of bFGF solution (20 µl) onto 2 mg of freeze-dried gelatin microspheres, which were then left at room temperature for 1 h. The solution (20 µl) was completely absorbed into the microspheres during swelling, because the solution volume was less than that theoretically required for the equilibrated swelling of microspheres.

Experimental Groups

Two weeks after surgery, the rabbits were randomly assigned to 1 of 4 experimental groups (n=11, each group) and treated for 4 weeks: group A, no treatment; group B,

supplemented with diet containing sarpogrelate (100 mg/kg per day); group C, single intramuscular injection of sustained-release form of bFGF microspheres (50 µg); group D: combined treatment with sustained-release bFGF and sarpogrelate.

Measurement of Hindlimb Blood Flow

With the use of non-radioactive colored microspheres (diameter, 15.5 ± 0.2 µm; Dye-trak, Triton Technology Ltd, Notts, UK), the resting regional hindlimb blood flow was determined in 5 rabbits from each experimental group.¹⁷ Six weeks after surgery, the reanesthetized rabbits were placed in the supine position on a temperature-controlled heating pad. Colored microspheres (3×10^6 particles) were injected into the left ventricle immediately before angiography. Reference arterial blood samples were collected through the aortic catheter, starting 10 s before the injection of the microspheres and continuing for 120 s at the rate of 2 ml/min. Two or more tissue specimens were obtained from the adductor muscle and the gastrocnemius muscle at the time of death. Samples were then placed into Teflon-sealed 16-ml screw-cap tubes and 7 ml of a 4 molar KOH solution containing 2% Tween 80 was added to each sample for digestion of the tissue. The photometric absorption of the resulting samples was measured using a diode array spectrophotometer (UV-1200, Shimadzu, Kyoto, Japan). The number of microspheres in each sample was calculated according to the optical density at the wavelength corresponding to dye color using standardization curves generated from known quantities of microspheres from the sample batch of spheres. Regional blood flow was then determined and expressed as ml/min per 100 g tissue.

Microangiographic Assessment

Six weeks after surgery, 6 rabbits from each experimental group were reanesthetized, and angiographic assessment was performed with synchrotron radiation microangiography with a spatial resolution of 30 µm.¹⁸ Contrast material containing 37% nonionic iodine (Iopamidol, Nihon Shering Co, Ltd, Osaka, Japan) was injected using an automated injector (Autoenhance A30-200, Nemoto Kyourindo Co, Ltd, Tokyo, Japan) at a rate of 1.25 ml/s via a 4F catheter placed immediately above the aortic bifurcation. Quantitative angiographic analysis of collateral vessel visualization was performed using a composite of 5×5 mm grids. The angiographic score was calculated as the ratio of grid intersections crossed by opacified arteries divided by the total number of grid intersections in the medial thigh region.¹⁹ This analysis was performed by a single observer who was unaware of the treatment regimen.

Histological Determination of Capillary Density

Immediately after the completion of the microangiographic study, the 24 rabbits were killed with an intravenous overdose of pentobarbital and potassium chloride. The hindlimbs were dissected and samples of the adductor muscle were removed for histological evaluation. After the samples were fixed in 10% formalin, followed by embedding in paraffin, they were frozen in liquid nitrogen for immunohistochemistry. The staining of endothelial cells was performed using a monoclonal antibody against CD31 (Dako Cytomation Co, Ltd, Glostrup, Denmark). Five fields from 6 different sections were randomly selected from each sample, and the number of capillaries was counted manually by 2 pathologists who were unaware of the grouping of

Fig 1. Effect of each treatment on resting hindlimb blood flow measured at 6 weeks after femoral artery extraction. The resting blood flow in the adductor muscles of the experimental (Top panel) and contralateral (Bottom panel) limbs was assessed by color microspheres and compared with the control group. bFGF, basic fibroblast growth factor.

Fig2. Effect of each treatment on capillary density examined at 6 weeks after femoral artery extraction. The capillary density in the adductor muscles of the experimental limbs was assessed histologically and compared with the control group. bFGF, basic fibroblast growth factor.

Fig3. Effect of each treatment on the growth and development of angiographically visible collateral vessels. The angiographic score represents the density of collateral vessels in the medial thigh region of the experimental limb. bFGF, basic fibroblast growth factor.

the rabbits. Finally, the capillary density was calculated as the number of capillaries/mm² of muscle.

Statistical Analysis

Results are expressed as the mean±standard deviation. Differences among groups were determined by 1-way analysis of variance followed by multiple comparisons using the Bonferroni/Dunn's test. All statistical analyses were performed using Statview™ software (Abacus, Calabosus, CA, USA). A p-value <0.05 was considered significant.

Results

There were no deaths of rabbits as a result of the surgical manipulation. We found neither local disorders nor complications attributable to the topical use of sustained-release bFGF.

Regional Blood Flow

The resting regional blood flow was assessed using the colored microspheres as described earlier. In the ischemic adductor muscle, the regional blood flow in group D was the highest among the 4 groups (4.0±0.7, 5.8±1.0, 7.2±0.6, 9.7±2.0 ml/min per 100 g tissue for groups A, B, C and D, respectively; p<0.01) (Fig 1). In addition, there was no statistically significant difference between the resting regional blood flow in the right adductor muscle (ischemic) and that in the left adductor muscle (non-ischemic) in group D (9.7±2.0 and 10.4±3.5 ml/min per 100 g tissue, respectively) (Fig 1). No significant differences in the normal hindlimb blood flow in the adductor muscles were observed among the 4 groups (8.6±2.4, 9.3±1.8, 8.8±1.5, 10.4±3.5 ml/min per 100 g tissue for each group) (Fig 1).

Capillary Density

A favorable effect of sustained-release bFGF was shown

Fig 4. Representative angiographic images of the experimental limb of rabbits treated with sarpogrelate (B), bFGF (C), or a combination of sarpogrelate and bFGF (D). Rabbits with no treatment served as the control (A). The diameter of the reference wire (arrowheads) was 130 μ m. Imaging field of each microangiogram was 20 $\times$ 20 mm. Note the increased density of collateral vessels in the medial thigh region of rabbits treated with sustained-release bFGF (C, D). A more increased diameter was observed in the rabbits with the combined treatment of bFGF and sarpogrelate (arrows). bFGF, basic fibroblast growth factor.

in terms of capillary proliferation (Fig 2). The adductor muscles of the ischemic hindlimbs were examined histologically 6 weeks after surgery. The mean values of the capillary density in groups treated with sustained-release bFGF were significantly higher than those in groups without bFGF (89 ± 17 , 96 ± 12 , 181 ± 19 , 179 ± 22 /mm² for groups A, B, C and D; $p < 0.01$). The mean value of the capillary density in the adductor muscle of the contralateral non-ischemic hindlimb was 129 ± 18 /mm². Thus, the capillary density in the ischemic hindlimb of rabbits treated with sustained-release bFGF was significantly higher than that in the non-ischemic hindlimb.

Microangiographic Assessment

Assessment of the number of angiographically visible collateral vessels (the aforementioned angiographic score) showed the highest increase in group D with the combined treatment among the 4 groups (0.41 ± 0.06 , 0.42 ± 0.04 , 0.68 ± 0.07 , 0.79 ± 0.06 for groups A, B, C and D, respectively; $p < 0.01$) (Fig 3).

Administration of sustained-release bFGF induced the enlargement of collateral vessels (Figs 4C,D). Importantly, a further vasodilatory effect was observed in the collateral vessels of the rabbits with then additional sarpogrelate treatment.

Discussion

This is the first study demonstrating that an angiogenic therapy aimed at collateral vessel growth and dilation significantly increased collateral blood flow. The results from the present study are in agreement with the conceptual framework of the functional improvement in collateral blood flow. Sustained-release bFGF enhanced angiogenesis and arteriogenesis, and sarpogrelate did not augment angiogenesis and arteriogenesis, but rather dilated collateral vessels with several layers of smooth muscle cells. Thus, angiogenesis, arteriogenesis and the dilation of collateral vessels contributed to augmented collateral blood flow in the rabbit hindlimb following femoral artery removal.

Data from the present study clearly demonstrate that

collateral blood flow was the highest in the rabbits with the combined treatment of sustained-release bFGF and sarpogrelate. Collateral blood flow is augmented mainly by arteriogenesis and the dilation of collateral vessels.³ Indeed, Hershey et al have elegantly shown that increases in collateral blood flow do not parallel angiogenesis, but rather arteriogenesis, in a rabbit hindlimb ischemia model! Also, it is well appreciated that vasodilators, such as nitroglycerin, dilate newly developed collateral vessels, and increase collateral blood flow.²⁰

For the evaluation of arteriogenesis, we used an angiographic score derived from microangiography, which allowed for the visualization of small collateral vessels with diameters greater than 30 μ m.^{18,19} The number of angiographically visible collateral vessels may be augmented not only by arteriogenesis, but also by vasodilation of smaller collateral vessels by vasodilatory agents. Thus, microangiograms indirectly reveal the conductance of collateral vessels. Regarding angiogenesis, our findings are intriguing, because the capillary density in the ischemic hindlimb showed a 1.4-fold increase compared with that in the non-ischemic hindlimb. However, it is unclear whether the enhanced angiogenesis favorably contributed to the functional regional blood flow in the ischemic muscle. Furthermore, because capillary density is largely affected by the volume of skeletal myocytes, the cross-sectional area of myocytes must be taken into account.

In the present study, we demonstrated that a single intramuscular injection of gelatin hydrogel microspheres incorporating bFGF (sustained-release bFGF) significantly improved tissue blood flow through angiogenesis and arteriogenesis in a rabbit hindlimb ischemic model. Therapeutic angiogenesis with growth factors has been developed for the treatment of severe PAD.^{7,8,19} Of the growth factors, bFGF is one of the most potent mitogens regulating the proteins that induce proliferation of a variety of cells and promote the growth and regeneration of organs and tissues *in vivo*.²¹ Although the biological half-life of bFGF in its free form is very short, it has been reported that collateral vessel growth can be obtained with multiple injections.⁸ In the present study, we used a single local injection of sus-

tained-release bFGF and from the significant increase in the number of capillaries and angiographically demonstrable collateral vessels, we confirmed that remarkable collateral growth through angiogenesis and arteriogenesis had occurred. In addition, the sustained-release bFGF induced significant regional blood flow recovery in the ischemic limbs. Because we did not find any local or systemic complications attributable to the topical use of sustained-release bFGF, this treatment would be applicable for critically ill patients with PAD. In the near future, the new treatment reported here may be comparable with other therapeutic modalities such as gene therapy,²² cell therapy with bone marrow cells²³ or peripheral blood mononuclear cells²⁴

It is tempting to speculate on the mechanisms for the effectiveness of sarpogrelate for enhancing the collateral blood flow in the present study. As shown in Fig 4, sarpogrelate dilated the collateral vessels, presumably by blockade of the 5-hydroxytryptamine (5-HT) subtype 2A receptor in the vascular smooth muscle cells in the newly developed collateral vessels. The dilation of collateral vessels by serotonin blockers, such as sarpogrelate and ketanserin, has been shown in humans with effort angina^{25,26}. Alternatively, the increased collateral blood flow mediated by sarpogrelate blockade of the 5-HT_{2A} receptor possibly augments shear stress at the site of preexistent collateral vessels. Indeed, arteriogenesis is potentiated through amplification of endogenous endothelial signaling mediated by changes in shear stress.^{27–29} It may be difficult to determine the relative contribution of sarpogrelate to acute vasodilation or the resultant chronically enhanced arteriogenesis. To this end, a microangiographic study of single administration of sarpogrelate may reveal the favorable vasodilatory effect of this serotonin blocker.^{25,26}

In summary, the present study demonstrated that intramuscular administration of a sustained-release form of bFGF stimulated collateral vessel growth, and combined treatment with sarpogrelate produced a further improvement in the hindlimb blood flow of a rabbit model of peripheral arterial insufficiency. These results suggest that the new therapy reported here could stimulate a meaningful improvement in collateral blood flow in patients with PAD.

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