Gelatin Hydrogel Microspheres Enable Pinpoint Delivery of Basic Fibroblast Growth Factor for the Development of Functional Collateral Vessels

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Background—Various growth factors promote collateral vessel development and are regarded as promising for the treatment of vascular occlusive diseases. However, an efficacious delivery system for them has yet to be established. We devised a strategy to augment functional collateral vessels by using acidic gelatin hydrogel microspheres (AGHMs) incorporating basic fibroblast growth factor (bFGF). The aim of the present study was to investigate the hypothesis that by intra-arterial (IA) administration of bFGF-impregnated AGHMs, bFGF could be delivered from AGHMs trapped in distal small-diameter vessels and thereby induce functional collateral vessels with an assured blood supply through the process of arteriogenesis.

Methods and Results—Various sizes of AGHMs (3 mg) incorporating $^{125}$I-labeled bFGF were injected into the left internal iliac artery of a rabbit model of hindlimb ischemia. Less than 50% of radioactivity accumulated in the ischemic hindlimb after injection of AGHMs that were 10 $\mu$m in diameter, whereas 80% of radioactivity was counted in the ischemic limb after administration of 29- or 59-$\mu$m-diameter AGHMs. Calf blood pressure ratio and the ratio of regional blood flow of the bilateral hindlimbs immediately before and after IA administration of 29-$\mu$m-diameter AGHMs showed no significant change. Then we evaluated the function of the developed collateral vessels 28 days after IA administration of bFGF-impregnated, 29-$\mu$m-diameter AGHMs. IA administration of bFGF-impregnated AGHMs induced marked collateral vessel improvement compared with IA administration of phosphate buffered saline–treated AGHMs and intramuscular administration of bFGF-impregnated AGHMs.

Conclusions—IA administration of bFGF-impregnated, 29-$\mu$m-diameter AGHMs strongly induced functional collateral vessels without worsening ischemia, indicating the possible therapeutic usefulness of this approach. (Circulation. 2004; 110:3322-3328.)

Key Words: angiogenesis ■ collateral circulation ■ growth substances ■ ischemia ■ microspheres

Local delivery of angiogenic growth factors augments collateral circulation in animal models of ischemia.1–5 However, there remain limitations in their therapeutic application with respect to safety and efficiency.6,7 Intra-arterial (IA) infusion of recombinant basic fibroblast growth factor (bFGF) was recently tested in randomized clinical trials and showed only limited effectiveness.8,9 The difficulty in the use of recombinant growth factors lies in their low accumulation in the ischemic tissue after IA administration and their rapid inactivation in vivo. We designed a method of site-specific and sustained delivery of bFGF by using acidic gelatin hydrogel microspheres (AGHMs). AGHMs have been developed for targeted delivery and controlled release of bFGF.10–12 After in vivo administration of bFGF-impregnated AGHMs, bFGF is continuously released, with biologic activity maintained during the biodegradation process of AGHM, and the kinetics of degradation can be controlled by altering the extent of gelatin cross-linking.10,11

To efficiently restore the blood supply to ischemic tissue, the developed collateral arteries need to be large-conductance vessels with mature wall structure and to have close connections with a bloodstream source. Therefore, it is desirable that the angiogenic factor involved in therapy can promote arteriogenesis, ie, the process of vessel maturation and enlargement.13–16 Furthermore, the ideal target of delivery should not be the tissue itself in the ischemic milieu but the donor artery, which refers to an artery with ample blood inflow that is located adjacent to the ischemic tissue and can potentially...
become an origin of collateral vessel development.\textsuperscript{17} Because angiogenic reactions ordinarily occur in small-diameter arteries,\textsuperscript{15,16} growth-promoting factors should be delivered to and act on the peripheral extremities of the donor artery.\textsuperscript{4} Our strategy was to develop functional collateral vessels by lodging bFGF-impregnated AGHMs in the peripheral part of the donor artery through IA administration. Trapped in small arterioles, AGHMs are expected to slowly release bFGF, a potent stimulator of arteriogenesis,\textsuperscript{15,16,18} thereby inducing large-conductance arteries with sufficient blood inflow toward the ischemic tissue.

The size of AGHMs is an important factor to achieve pinpoint delivery, because if the size of the AGHMs is too small, they pass through the capillary network and enter the venous system,\textsuperscript{19} and if they are too large, they are likely to be trapped in more proximal branches, possibly worsening distal perfusion.\textsuperscript{20} In the present study, we first determined the optimal size of AGHMs and then evaluated the function of the developed collateral vessels promoted by IA administration of bFGF-incorporating AGHMs. The delivery period of bFGF was adjusted to \(\approx 14\) days, because previous studies had shown that delivery of angiogenic growth factors for 14 days was sufficient to promote favorable development of collateral vessels in ischemic tissues.\textsuperscript{1,4}

**Methods**

**Preparation of AGHMs**

AGHMs with mean diameters of 10 (10.0±3.7), 29 (29.3±10.7), 59 (59.4±19.5), and 75 (75.1±18.1) \(\mu\)m were prepared from gelatin with an isoelectric point of 4.9 (Nitta Gelatin) as described previously.\textsuperscript{10,11} The length of time for degradation of AGHMs was adjusted to 14 days. Then, 100 \(\mu\)g human recombinant bFGF (trafermin, 154 amino acid residues, 17.1 kDa; Kaken Pharmaceutical) in 30 \(\mu\)L phosphate-buffered saline (PBS) was added to 3 mg dry AGHMs to obtain bFGF-impregnated AGHMs. Some AGHMs were treated with PBS alone as a vehicle control.

**Animal Model of Hindlimb Ischemia**

The present study used a rabbit model of chronic hindlimb ischemia in all experiments. Male Japanese White rabbits weighing 3.0 to 3.5 kg (Saitama Rabbitry, Saitama, Japan) were anesthetized with an intramuscular (IM) injection of a mixture of ketamine (50 mg/kg) and xylazine (2.5 mg/kg). The left femoral artery was completely excised from its origin just above the inguinal ligament to the bifurcation of the saphenous and popliteal arteries.\textsuperscript{2,21} At 21 days after femoral artery excision, animals were treated with AGHMs. Before sample collection, animals were euthanized by intravenous injection of 150 mg pentobarbital. All protocols conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

**Administration of AGHMs**

For IA administration, AGHMs were suspended in 5.0 mL PBS and injected into the left internal iliac artery (IIA) of the rabbits, which represents the donor artery of the model, through a 3F end-hole catheter introduced into the carotid artery. The tip of the catheter was positioned in the IIA at the level of the interspace between the seventh lumbar and first sacral vertebrae under fluoroscopic guidance. For IM administration, AGHMs were suspended in 0.9 mL PBS and injected into 6 different sites in the left medial thigh muscles.

**Distribution of Administered bFGF-Impregnated AGHMs**

Radiolabeling of bFGF with \(^{125}\)I was performed according to the lactoperoxidase method.\textsuperscript{22} Various sizes of AGHMs (10, 29, or 59 \(\mu\)m in diameter) incorporating \(^{125}\)I-labeled bFGF were administered IA into the left IIA (\(n=4\) each). Five hours later, the above-knee and below-knee muscles were taken from the bilateral hindlimbs, and the internal organs (liver, kidneys, heart, lungs, spleen, and testes) were also resected. Each sample was weighed (\(W_{\text{WHOLE}}\)) and homogenized. A certain volume of each homogenate was weighed (\(W_{\text{SAMPLE}}\)) and counted with a gamma counter (\(C_{\text{SAMPLE}}\)). The radioactivity of each sample was calculated as \(C_{\text{SAMPLE}}/W_{\text{SAMPLE}}\). The ratio of the radioactivity of each to the total was calculated. The radioactivity distribution 5 hours after IM administration and 7 days after IA administration of 29-\(\mu\)m-diameter AGHMs incorporating \(^{125}\)I-labeled bFGF was also evaluated (\(n=4\) each).

**Short-Term Changes in Tissue Perfusion After IA Injection of AGHMs**

Calf blood pressure ratio and regional blood flow (RBF) of the bilateral hindlimb muscles were measured before and after IA injection of PBS-treated 29-, 59-, or 75-\(\mu\)m-diameter AGHMs into the left IIA (\(n=6\) each). Calf blood pressure ratio was defined as the ratio of systolic pressure of the left hindlimb to that of the right. RBF was analyzed with the use of colored microspheres\textsuperscript{3,23} (diameter, 15 \(\mu\)m; Dye-Trak, Triton Technology). Colored microspheres (\(3\times10^3\) particles) were injected into the left ventricle through a catheter inserted into the right common carotid artery. A reference blood sample was collected at a rate of 2.0 mL/min for 3 minutes from the descending thoracic aorta through another catheter inserted into the left common carotid artery. Yellow and eosin microspheres were used before and after injection of AGHMs, respectively. The entire crural muscles and distal half of the thigh muscles were collected, and RBF of the bilateral hindlimbs before and after AGHM injection was calculated by spectrophotometry. The ratio of RBF of the left hindlimb to that of the right was calculated.

**Process of AGHM Lodging**

AGHMs, 29 \(\mu\)m in diameter, which incorporated 100 \(\mu\)g bFGF, were administered IA into the left IIA. Transverse tissue sections (5 \(\mu\)m thick) were cut from the left semimembranous and great adductor muscles 7 days after AGHM injection. The sections were treated with hematoxylin and eosin stain and immunostaining for macrophages (RAM11, DAKO).

**Evaluation of Collateral Vessel Development**

AGHMs, 29 \(\mu\)m in diameter, which incorporated 100 \(\mu\)g bFGF, were administered into the left IIA (IA-FGF group, \(n=8\)). Other sets of animals were subjected to the following 3 procedures as controls: IA injection of PBS-treated AGHMs into the left IIA (IA-PBS group, \(n=8\)); IM injection of bFGF-impregnated AGHMs into the left thigh muscles (IM-FGF group, \(n=8\)); and IM injection of PBS-treated AGHMs into the left thigh muscles (IM-PBS group, \(n=8\)). Normal rabbits untreated by any means were also evaluated for comparison (\(n=5\)).

Evaluations of collateral vessel development were carried out 28 days after AGHM administration. After the determination of calf blood pressure ratio, a 0.014-in. Doppler guidewire (Endo Sonics) was introduced into the proximal part of the left IIA. Average peak velocity was measured at rest, maximum average peak velocity was determined after injection of 2 mg papaverine, and then in vivo blood flow of the left IIA was calculated from both average peak velocity measurements as described previously.\textsuperscript{3,4} Subsequently, a 3F end-hole catheter was introduced into the left IIA. Angiograms were taken as described previously, and the angiographic score was determined.\textsuperscript{3,4} Collateral conductance was measured with the use of colored microspheres.\textsuperscript{3,4} Animals were intubated through a tracheotomy and ventilated with room air. The jugular vein was cannulated for continuous infusion of lactated Ringer’s solution and occasional injections of pentobarbital to maintain an appropriate level of anesthesia.
anesthesia. After heparinization with 2000 U heparin, the infrarenal abdominal aorta was exposed, cut down, and cannulated in proximal and distal directions with 14-gauge catheters (i.d., 1.73 mm), which were connected to a pump to maintain a stable flow to the hindlimbs. The proximal pressure (PP) of collateral arteries was monitored in the distally directed catheter. The left saphenous artery was also cannulated, and distal pressure of collateral arteries (DP) was monitored. Disodium ATP was continuously infused to the pump-driven circuit (1 mg \( \cdot \) kg\(^{-1} \cdot \) min\(^{-1} \)) to achieve maximum vasodilation, and then RBF of the left hindlimb was measured at 2 different proximal pressure levels (\( \approx 50 \) and 60 mm Hg) by using microspheres of different colors (yellow and eosin). A reference blood sample was collected at a rate of 0.6 mL/min from the right femoral artery through a catheter. Collateral conductance was calculated as 100 \( \frac{\text{RBF}}{\text{PP}} \), and the mean value of collateral conductance estimated under the 2 PP levels was used for statistical analysis.

Histologic Study
Transverse tissue sections (5 \( \mu \)m thick) were cut from the left semimembranous muscle 28 days after AGHM injection. Capillary endothelial cells were stained by the indoxyl tetrazolium method, and capillary density was measured as described previously. The sections were also immunostained for \( \alpha \)-SM actin (1A4, DAKO), and the number of vessels surrounded by SMCs was counted in each section to determine the SMC-positive vessel density.

Statistical Analysis
Data are presented as mean±SD. Statistical significance was evaluated by Student’s \( t \) test for comparisons between 2 means and the Tukey-Kramer method for multiple comparisons. All data were considered significant at \( P<0.05 \).

Results
Distribution of Administered bFGF-Impregnated AGHMs
Five hours after IA injection of 10-\( \mu \)m-diameter AGHMs, <50% of the radioactivity had accumulated in the ischemic limb, whereas abundant activity was detected in the liver, kidneys, and lungs (Figure 1A). In contrast, 77% to 78% of radioactivity was counted in the ischemic limb after IA administration of 29- and 59-\( \mu \)m-diameter AGHMs (Figure 1B and 1C). The radioactivity distribution after injection of 29-\( \mu \)m-diameter AGHMs was similar to that after injection of 59-\( \mu \)m-diameter AGHMs. Five hours after IM administration of 29-\( \mu \)m-diameter AGHMs, \( \approx 99\% \) of radioactivity was detected in the ischemic limb muscles (Figure 1D). Seven days after IA injection of 29-\( \mu \)m-diameter AGHMs, \( \approx 73\% \) of radioactivity was still detected in the ischemic limb (Figure 1E).

Short-Term Changes in Tissue Perfusion After IA Injection of AGHMs
In animals that received 29-\( \mu \)m-diameter AGHMs, no significant change was detected in the calf blood pressure ratio or in the ratio of RBF of the left hindlimb to that of the right before versus after AGHM administration. A marked decrease of blood perfusion was observed in animals treated with 59- or 75-\( \mu \)m-diameter AGHMs in both evaluations (Figure 2A and 2B).

Process of AGHM Lodging
The vessel where AGHMs had lodged showed occlusive change 7 days after IA administration (Figure 3A). There was no apparent accumulation of macrophages around AGHMs (Figure 3B).

Evaluation of Collateral Vessel Development
Calf blood pressure ratio showed significant improvement in the IA-FGF group compared with the IA-PBS and IM-FGF groups.
Capillary density was also significantly increased in the IA-FGF group compared with the IA-PBS and IM-FGF groups. These values in the IM-FGF group were also significantly higher than those in the IM-PBS group (Figure 4A and 4B). Resting blood flow and maximum blood flow of the IIA demonstrated a significant increase of collateral blood perfusion in the IA-FGF group compared with the IA-PBS and IM-FGF groups (Figure 4C). Resting blood flow of the IIA was significantly higher in the IM-FGF group than in the IM-PBS group, whereas no significant difference was observed in maximum blood flow of the IIA between the 2 groups (Figure 4C).

Collateral conductance was significantly higher in the IA-FGF group than in the IA-PBS and IM-FGF groups, whereas the value in the IM-FGF group showed significant improvement in comparison with the IM-PBS group (Figure 4D). Selective internal iliac arteriography 28 days after AGHM injection showed marked collateral vessel development in the IA-FGF and IM-FGF groups, whereas few collateral vessels were observed in the IA-PBS and IM-PBS groups (Figure 5A–5E). The angiographic score was significantly higher in the IA-FGF group than in the IA-PBS and IM-FGF groups. Significant improvement was detected in the IM-FGF group compared with the IM-PBS group (Figure 5F). In the IA-FGF group, a greater number of vessels with SMCs around them was observed (Figure 6A–6E). SMC-positive vessel density was significantly higher in the IA-FGF group compared with the IA-PBS and IM-FGF groups, with no difference observed between the IM-FGF and IM-PBS groups (Figure 6F).

Discussion
In the present study, we demonstrated the efficacy of a new delivery system of bFGF through the use of AGHMs. AGHMs, 29 μm in diameter and incorporating bFGF, showed high accumulation in the chronically ischemic hindlimb of a rabbit model after IA administration to the donor artery, without further deterioration of blood perfusion. They induced functional collateral circulation more potently through IA injection than through IM injection.

Apart from particular situations in which an ischemic lesion is caused only by microvascular changes, a certain volume of the capillary bed remains preserved, even in ischemic tissues. The principal goal of angiogenic therapy is to develop collateral vessels that can provide sufficient blood flow to the preexisting capillary network in the ischemic tissues. Thus, a desirable collateral artery is a conduit with a large diameter connecting the source of blood flow to this capillary bed, even though it is also beneficial to induce additional development of capillaries in the ischemic tissues. To achieve the ideal collateral circulation described, the choice of growth factor used in therapy and the choice of delivery target are the most important factors.

There are 3 forms of angiogenic reactions in vivo: vasculogenesis, angiogenesis, and arteriogenesis. Vasculogenesis in adults represents the differentiation and replication of endothelial progenitor cells to form a primitive vasculature. Angiogenesis is the process of endothelial proliferation and migration from preexisting capillaries to form a new vascular network. Thus, vessels newly formed by the process of vasculogenesis and angiogenesis consist mainly of endothelial cells and are therefore small, fragile arteries. In contrast, arteriogenesis refers to the maturation and stabilization process of preexisting arterioles, which includes SMC recruitment and vessel enlargement through vascular remodeling. Therefore, the growth factor used for therapy should have a potent effect to induce arteriogenesis to acquire large-conductance collateral vessels, although induction of vasculogenesis and/or angiogenesis might also be advantageous. It was shown by Deindl et al that bFGF plays an important role during adaptive arteriogenesis, and it also promotes angiogenesis in ischemic tissues. We thus believe that bFGF is one of the most suitable growth factors for angiogenic therapy.

For development of functional collateral arteries by arteriogenesis, bFGF has to be delivered to small, preexisting, communicating vessels between the extremities of the donor artery and the vascular network in the ischemic tissue (Figure 7A). Even though these communicating vessels would be too small to function before therapy, site-specific delivery of
bFGF might cause small vessels to grow into functional collateral arteries (Figure 7B), and the same location is also ideal for the process of angiogenesis (Figure 7C).

In the present study, the radioactivity distribution pattern 5 hours after IA injection of 10-μm–diameter AGHMs through the left IIA demonstrated that only about half of the radioactivity had remained in the ischemic limb. The result suggested that some AGHMs flowed into the venous system and systemic circulation. In contrast, ∼80% of radioactivity accumulated in the ischemic hindlimb after IA administration of 29- or 59-μm-diameter AGHMs. These 2 distribution patterns showed a strong resemblance. Most of the radioactivity detected in the liver, kidneys, and lungs after injection of 29- and 59-μm-diameter AGHMs could possibly be attributed to free, 125I-labeled bFGF, because ∼30% of bFGF cannot be incorporated into AGHMs through the sorption process.10,11 Thus, most 29- and 59-μm-diameter AGHMs were considered to have been captured in the distal extremities of the IIA. On the other hand, we verified that 3 mg AGHMs with a diameter of 29 μm had little influence on the preexistent collateral circulation after IA injection, whereas blood perfusion was significantly impaired after IA injection of 59-μm-diameter AGHMs. The high accumulation of radioactivity 7 days after administration of 29-μm-diameter AGHMs indicated that a substantial amount of bFGF remained in the ischemic limb even at that time. The radioactivity distribution after IM administration of AGHMs incorporating radiolabeled bFGF suggested that most of the AGHMs had remained around the injection site (Figure 1D). However, collateral vessel development in the IM-FGF group was less pronounced than that in the IA-FGF group, indicating that AGHMs delivered bFGF more effectively to the target vessels through IA administration than through IM injection. From these results, we assume that even though AGHMs caused minimal microembolism after IA injection, as demonstrated by histologic findings (Figure 3), the released bFGF could spread to the surrounding circulatory network, inducing collateral vessels toward the ischemic tissue. Our data indicated that AGHMs could serve as a safe and efficacious carrier to realize pinpoint delivery of bFGF to targeted sites through IA administration when the size and quantity of AGHMs are appropriate.

The evaluation methods performed to assess collateral vessel development in the present study have been frequently used and established in several previous studies.2–4 Calf blood pressure ratio and in vivo blood flow of the IIA are conventional parameters representing the general degree of collateral vessel development. Capillary density of ischemic muscle is a param-
eter of angiogenesis and vasculogenesis, because it is based on staining of endothelial cells. These parameters revealed superior efficacy of IA injection of bFGF-impregnated AGHMs in promoting collateral vessel augmentation compared with IM injection. Blood flow of the IIA in normal rabbits was lower than in the ischemic models, because the left femoral artery was preserved in normal rabbits. In contrast, the angiographic score, collateral conductance, and SMC-positive vessel density can be considered parameters of arteriogenesis. Collateral conductance reflects the function of solid collateral vessels to conduct blood flow, and mature vessels possess a medial layer containing SMCs. Thus, these 3 parameters represent the extent of mature and functional collateral vessel development through the process of arteriogenesis. In the present study, all of these parameters showed the superiority of IA injection of bFGF-impregnated AGHMs in the induction of large-conductance collateral vessels compared with IM injection of the same material. In the IA-FGF group, collateral conductance reached about half the value of normal rabbits, and the angiographic score was 3 times that in normal animals. These results suggest that arteriogenesis played a predominant role in the growth of collateral vessels as promoted by IA administration of bFGF-impregnated AGHMs. IM administration could possibly augment the vascular bed in ischemic tissues, but the induced collateral vessels might possess less communication with the donor artery, resulting in poorer function for blood supply. Although we could not compare the amount of bFGF protein in the collateral vessels of each experimental group and the exact mechanism of vessel growth after growth factor delivery remains unclear, the aforementioned results suggest the efficacy of our delivery strategy.

In summary, the present study demonstrated the therapeutic potential of bFGF-impregnated, 29-μm-diameter AGHMs by IA administration. Because the biosafety of gelatin has been proven through broad clinical use and the injected AGHMs disappear by
degradation when the release of bFGF is finished, it is likely that our strategy can be safely used in clinical applications. Although the time of treatment should be investigated further, the results of this study suggest another option for the treatment of vascular occlusive diseases.

References


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