Therapeutic site selection is important for the successful development of collateral vessels

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Background: **Induction of collateral development to improve tissue perfusion is a promising approach for the treatment of arterial occlusive diseases. Several growth factors and cells have been reported to increase collateral circulation; however, the appropriate site for the delivery of these factors and cells is unclear. In this study, we identified the delivery site for growth factor in a rabbit model of limb ischemia and evaluated whether specific delivery of basic fibroblast growth factor (bFGF) to this site enhanced collateral augmentation.**

Methods: **The left femoral artery of Japanese white rabbits was excised to induce limb ischemia. Twenty-eight days thereafter, angiograms were obtained to identify the typical pattern of collateral development in this model. Subsequently, bFGF (100 μg) was selectively injected into the left coccygeofemoral muscle (coccygeo group) or adductor muscle (adductor group), major thigh muscles in proximity. Collateral development was evaluated at 28 days after injection, and its mechanism was assessed by immunologic and morphometric analyses of muscle samples.**

Results: **Angiographic evaluation of this model revealed that after femoral artery excision, collateral vessels generally developed in the left coccygeofemoral muscle, whereas few collateral vessels were detected in the left adductor muscle. At 28 days after injection, calf blood pressure ratio, defined as left pressure to right pressure, was significantly higher in the coccygeo group than in the adductor group (0.85 ± 0.05 vs 0.69 ± 0.05, respectively; $P < .01$). Similar results were observed in blood flow through the internal iliac artery (resting: 24.6 ± 6.1 vs 17.4 ± 8.0 mL/min, $P < .01$; maximum: 47.4 ± 12.3 vs 33.2 ± 10.7 mL/min, $P < .01$) and in the angiographic score (0.67 ± 0.13 vs 0.39 ± 0.11; $P < .01$). Immunologic analyses of the coccygeofemoral muscle at day 3 showed marked expressions of Ki-67, monocyte chemotactic protein 1, and FGF receptor 1 in the coccygeo group compared with the adductor group. Morphometric analyses of the same muscle at day 14 also revealed that collateral vessel density and wall thickness were significantly increased in the coccygeal group compared with the adductor group.**

Conclusions: **These findings demonstrated that selective bFGF delivery to the coccygeofemoral muscle markedly improved collateral development and limb perfusion compared with delivery to the adductor muscle, suggesting that site selection is important in increasing therapeutic efficacy. (J Vasc Surg 2015;62:190-9.)**

Clinical Relevance: **Several clinical trials of angiogenic therapy for the treatment of peripheral arterial diseases have been conducted. In these trials, angiogenic factors or cells were delivered by intra-arterial or intramuscular injection. However, wide dispersion of the delivered substances to distal areas occurs with intra-arterial injections, and intramuscular injections usually involve broad, multiple injections to the ischemic limb; therefore, both methods might fail to achieve appropriate target site delivery for collateral development. On the basis of the conclusions of this study, accurate delivery of angiogenic substances to the appropriate site could markedly enhance the therapeutic efficacy of human angiogenic therapy.**

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Induction of angiogenic reactions to improve tissue perfusion is a promising approach for the treatment of arterial occlusive diseases, and the most important mechanism of angiogenic therapy is considered to be the enhancement of collateral circulation to the ischemic lesion. Although a variety of strategies for effective angiogenic therapies have been presented, the basic concept in most of these strategies is the local delivery of bioactive factors or cells that potentially promote angiogenic processes in vivo.[1-4] Previous studies have attempted to deliver several bioactive factors or cells in animal models of ischemia and reported favorable therapeutic effects of some growth factors or cells, such as basic fibroblast growth factor (bFGF),[5-7] vascular endothelial growth factor,[2,8-11] and bone marrow mononuclear

cells.[12-14] Those studies have successfully identified growth factors or cells that are adequate for delivery substances in angiogenic therapy; however, the appropriate site for such factors or cells is currently unclear. Recently, several clinical trials testing angiogenic therapies have been carried out.[15-19] In most of these trials, angiogenic factors or cells were delivered by intra-arterial injection or intramuscular injection. When angiogenic substances are administrated by an intra-arterial injection, they disperse widely to areas distal to the injection site and might fail to specifically target areas that are appropriate for collateral vessel formation. When substances are delivered by intramuscular injection, the procedure is usually broad, multiple injections to the muscles of the ischemic limb, which might also increase the possibility of irrelevant delivery. Indeed, large-scale clinical trials testing these substances have shown limited therapeutic efficacy.[15-19] We hypothesized that one reason for poor outcome might be the potential inefficiency of the delivery. If the delivery site for angiogenic therapy can be clearly identified, it might be possible to notably increase the therapeutic efficacy by specific delivery to this region.

The purpose of this study was to identify the therapeutic site for injection of the growth factor for the most effective development of collateral vessels in a rabbit model of chronic limb ischemia.

METHODS

Animal model of chronic limb ischemia. This study used a rabbit model of chronic limb ischemia to evaluate the development of collateral vessels. Reliable methods to evaluate the perfusion of rabbit limb have been previously reported, and several previous studies testing angiogenic therapies have used the rabbit model of limb ischemia.[2,6,20] Male Japanese white rabbits weighing 2.5 to 3.0 kg (Saitama Rabbitry, Saitama, Japan) were anesthetized with an intramuscular injection of a mixture of ketamine (50 mg/kg) and xylazine (2.5 mg/kg). The left femoral artery was completely excised from its proximal origin to the bifurcation formed by the saphenous and popliteal arteries. At 28 days after femoral artery excision, the left hind limb of the rabbits developed chronic ischemia.[6,7,10,20] All protocols conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 84-23, revised 1996).

Anatomic analyses of collateral development in the rabbit model of limb ischemia. To clarify the anatomic pattern of collateral development in the rabbit model of limb ischemia, we carried out aortic angiography at 28 days after femoral artery removal. Immediately after an overdose injection of pentobarbital, an 18-gauge polyethylene infusion catheter (Terumo, Tokyo, Japan) was introduced into the abdominal aorta in the distal direction, and a mixed solution of lead oxide (20 g; Wako, Tokyo, Japan), gelatin (0.3 g; Wako), and water (10 mL) was injected. Because lead oxide is a radiocontrast reagent and has a vivid orange color, the arteries containing the mixed solution are recognizable under a fluoroscope and also visible from the outside when the arteries are exposed. With the fluoroscopic image as reference, we dissected the collateral vessels by using the orange color as a guide and analyzed the anatomic pattern of collateral development in this model. This experiment was repeated three times.

Administration of bFGF. To investigate if the delivery site of bFGF influences the development of collateral vessels in the rabbit model of chronic limb ischemia, we injected bFGF (trafermin, human recombinant bFGF; Kaken Pharmaceutical, Tokyo, Japan) in either the left coccygeofemoral muscle (coccygeo group) or the left adductor muscle (adductor group) at 28 days after femoral artery removal, when a stable, chronic state of limb ischemia has developed.[21,22]

The left coccygeofemoral muscle is the region where the collateral vessels are expected to develop in this model, and the location of this muscle could be identified through the buttock skin because the outline of it appears clearly by moistening of the skin. In contrast, few collateral vessels are expected to develop in the left adductor muscle. In the coccygeo group, 100 μg of bFGF in 100 μL of phosphate-buffered saline (PBS) was injected into three different sites in the left coccygeofemoral muscle, and 100 μL of PBS alone was injected into three different sites in the left adductor muscle. In the adductor group, 100 μg of bFGF in PBS was injected into three different sites in the adductor muscle, and 100 μL of PBS alone was injected into three different sites in the coccygeofemoral muscle. The dose of bFGF (100 μg) was chosen on the basis of previous studies in which bFGF protein was administered for the treatment of limb ischemia.[6,16] As a control, another set of rabbits with chronic limb ischemia received 100 μL of PBS in both the coccygeofemoral muscle and adductor muscle (vehicle group).

Evaluation of collateral development. Collateral vessel development was evaluated at 28 days after the administration of bFGF/PBS (coccygeo group, n = 9; adductor group, n = 9; vehicle group, n = 10), a period evaluated in previous studies with the rabbit model.[6,7,20] First, the calf blood pressure was measured in both hind limbs, and the calf blood pressure ratio, defined as the ratio of left systolic pressure to right systolic pressure, was calculated. This measurement was also carried out immediately before the administration of bFGF/PBS.

After the measurement of calf blood pressure, a 3F end-hole catheter was introduced into the left iliac artery through the carotid artery, and a 0.014-inch Doppler guidewire (EndoSonics, Rancho Cordova, Calif) was introduced through the 3F catheter to the proximal part of the left internal iliac artery. The average peak velocity was measured at rest, and then the maximum average peak velocity was determined after the injection of 2 mg of papaverine (Dainippon Pharmaceutical, Osaka, Japan). In vivo blood flow was calculated as previously described.[6,7,20] Angiograms were then taken, and the angiographic score was determined as described previously.[6,7,20,21] Briefly, angiographic score was determined by a grid overlay composed of 2.5-mm-diameter circles arranged in rows spaced 5 mm apart. This overlay was placed over the angiogram, recorded for 4 seconds, at the level of the medial thigh.