

Adequate Selection of a Therapeutic Site Enables Efficient Development of Collateral Vessels in Angiogenic Treatment With Bone Marrow Mononuclear Cells

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Background—Induction of angiogenic mechanisms to promote development of collateral vessels is considered promising for the treatment of peripheral arterial diseases. Collateral vessels generally develop from preexisting arteriolar connections, bypassing the diseased artery. We speculated that induction of angiogenic mechanisms should be directed to such arteriolar connections to achieve efficient collateral development. The aim of this study was to verify this hypothesis using autologous transplantation of bone marrow mononuclear cells in the rabbit model of chronic limb ischemia.

Methods and Results—The left femoral artery was excised to induce limb ischemia in male rabbits. In this model, arteriolar connections in the left coccygeofemoral muscle tend to develop into collateral vessels, although this transformation is insufficient to alleviate the limb ischemia. In contrast, arteriolar connections in the closely located adductor muscle do not readily develop into collateral vessels. At 21 days after ischemia initiation, a sufficient number of automononuclear cells were selectively injected in the left coccygeofemoral muscle (coccygeo group) or left adductor muscle (adductor group). Evaluation of calf blood pressure ratios, blood flow in the left internal iliac artery, and angiographic scores at day 28 after injection revealed that collateral development and improvement of limb ischemia were significantly more efficient in the coccygeo group than in the adductor group. Morphometric analysis of the coccygeofemoral muscle at day 14 showed similar results.

Conclusions—Specific delivery of mononuclear cells to the coccygeofemoral but not the adductor muscle effectively improves collateral circulation in the rabbit model of limb ischemia and suggests that adequate site selection can facilitate therapeutic angiogenesis. (*J Am Heart Assoc.* 2015;4:e002287 doi: 10.1161/JAHA.115.002287)

Key Words: arteriogenesis • collateral circulation • ischemia

Induction of angiogenic mechanisms to enhance regional blood perfusion, also known as *therapeutic angiogenesis*, is a promising approach for the treatment of peripheral arterial diseases.¹ A variety of strategies that have been proposed to achieve effective therapeutic angiogenesis share a basic

framework of local delivery of cells or bioactive factors that could promote angiogenic mechanisms *in vivo*.^{2,3} Previous studies have tested several cell types and factors in animal models of limb ischemia, and abundant evidence has been accumulated regarding which cells or factors are effective when delivered.⁴ Autologous transplantation of bone marrow mononuclear cells (MNCs) is a famous cell delivery protocol, and potent therapeutic effects of bioactive factors such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), and other angiogenic growth factors have been shown on delivery.^{5–8} Considerable progress has been made in the development of delivery methods, especially for angiogenic factors, and sophisticated procedures have been created by applying novel techniques such as gene transfer and drug delivery systems.⁷ Previous studies, however, presented only limited evidence with respect to which sites are adequate for delivery of cells or factors. Although some large-scale clinical trials have been conducted to test therapeutic angiogenesis, the reported therapeutic efficacies

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have been limited.^{5,9} We hypothesized that a reason for the limited outcomes of these clinical trials might be a suboptimal choice of delivery site.

Augmentation of collateral circulation directed to ischemic lesions is the essential mechanism that improves blood perfusion in therapeutic angiogenesis. Collateral vessels generally develop from arteriolar connections that are present in several tissues and organs.^{9–11} Arteriolar connection is a microvascular channel that connects different arterial trees at the peripheral level. When the proximal part of an arterial tree is occluded, the other arterial tree partially supplies the downstream perfusion area via the arteriolar connections, thereby preventing or alleviating ischemia. The arteriolar connections then tend to develop into collateral vessels. If this collateral development is not sufficiently robust, chronic ischemia may occur in the distal perfusion area, and that is the condition to be treated. Consequently, the expected purpose of therapeutic angiogenesis might be to facilitate the growth of underdeveloped arteriolar connections into well-developed collateral vessels. Based on these considerations, underdeveloped arteriolar connections may represent a promising site for delivery of angiogenic substances.

Our previous investigation of the anatomic route of collateral vessel development in the rabbit model of limb ischemia revealed that arteriolar connections in the coccygeofemoral muscle tended to develop into collateral vessels, although this growth was insufficient in most cases (Figure 1).¹² Based on this finding, we hypothesized that the arteriolar connections in the coccygeofemoral muscle are an adequate delivery site for effective collateral development. In the present study, this hypothesis was tested by selective delivery of autologous MNCs to the coccygeofemoral muscle in the rabbit model, followed by evaluation of collateral vessel development and limb perfusion. MNC delivery to proximal muscle served as a control.

Materials and Methods

Animal Model of Chronic Limb Ischemia

In the present study, we used a rabbit model of chronic limb ischemia. 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. Chronic ischemia developed in the left hind limb by day 21 after femoral artery excision. All protocols conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health publication No. 84-23, revised 1996).

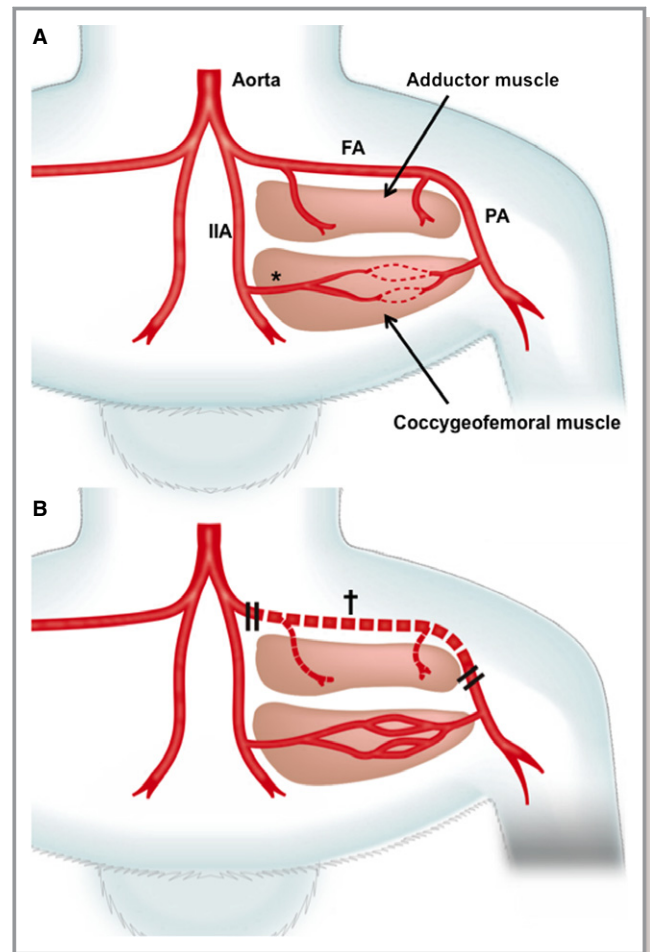


Figure 1. A, The coccygeofemoral muscle receives blood supply from the posterior gluteal artery and the branches of the PA. These 2 arterial systems are linked at the peripheral level by arteriolar connections. Although the adductor muscle is located close to the coccygeofemoral muscle, its feeding arteries are the deep femoral arteries and the branches of the PA. B, When the FA is removed, the arteriolar connections in the coccygeofemoral muscle tend to develop into collateral vessels that conduct blood flow from the posterior gluteal artery to the ischemic limb via the PA; however, this mechanism is insufficient to compensate for the ischemia. The expected purpose of therapeutic angiogenesis is to stimulate the growth of the underdeveloped arteriolar connections. FA removal promotes little development of collateral vessels in the adductor muscle because few arteriolar routes are available to bypass the FA. *Posterior gluteal artery, †Excision of the left FA. FA indicates femoral artery; IIA, internal iliac artery; PA, popliteal artery.

Aspiration and Isolation of MNCs

MNCs were harvested from the rabbits at day 21 after femoral artery removal. Overall, 5 mL of bone marrow was aspirated under anesthesia from right iliac crest through an infant aspiration needle (Terumo). The MNC fraction was isolated from aspirated bone marrow by centrifugation in Histopaque density gradient (Sigma-Aldrich), as described previously.¹³

Distribution of MNCs After Injection in the Coccygeofemoral Muscle

The obtained MNCs (5×10^6 cells) were labeled with a green fluorescent marker using a labeling kit (PKH2 Green Fluorescent Cell Linker Kit; Sigma-Aldrich) and immediately transplanted to the left coccygeofemoral muscle of the same rabbit, as described previously (Figure 2A).¹⁴ Briefly, the labeled MNCs were suspended in 1 mL phosphate buffered saline (PBS), and equal volumes were injected with a 25-gauge needle in the coccygeofemoral muscle at 3 different sites. The outline of the coccygeofemoral muscle was clearly identified through the moistened buttock skin. The 3 injection sites were set at equal intervals on the longitudinal median line of the muscle, and the needle tip was set at a depth of 10 mm from skin surface. At days 3 ($n=3$) and 7 ($n=3$) after transplantation, the coccygeofemoral muscle and the adductor muscle (a major muscle proximal to the coccygeofemoral

muscle) were excised and divided transversely into 8 samples, which were frozen at -80°C . These frozen samples were cut in $4\text{-}\mu\text{m}$ -thick sections and analyzed using fluorescent microscopy.

Autologous Transplantation of MNCs in the Coccygeofemoral or Adductor Muscle

Model animals were assigned to 3 study groups (Figure 2B): animals receiving auto-MNCs in the left coccygeofemoral muscle (coccygeo group, $n=10$), animals receiving auto-MNCs in the left adductor muscle (adductor group, $n=10$), and control animals receiving no MNCs (vehicle group, $n=10$). The coccygeofemoral muscle contains arteriolar connections that tend to develop into collateral vessels in this rabbit model, whereas few collateral vessels are expected to develop from arteriolar connections in the adductor muscle (Figure 1).¹² At day 21 after excision of the left femoral artery, MNCs

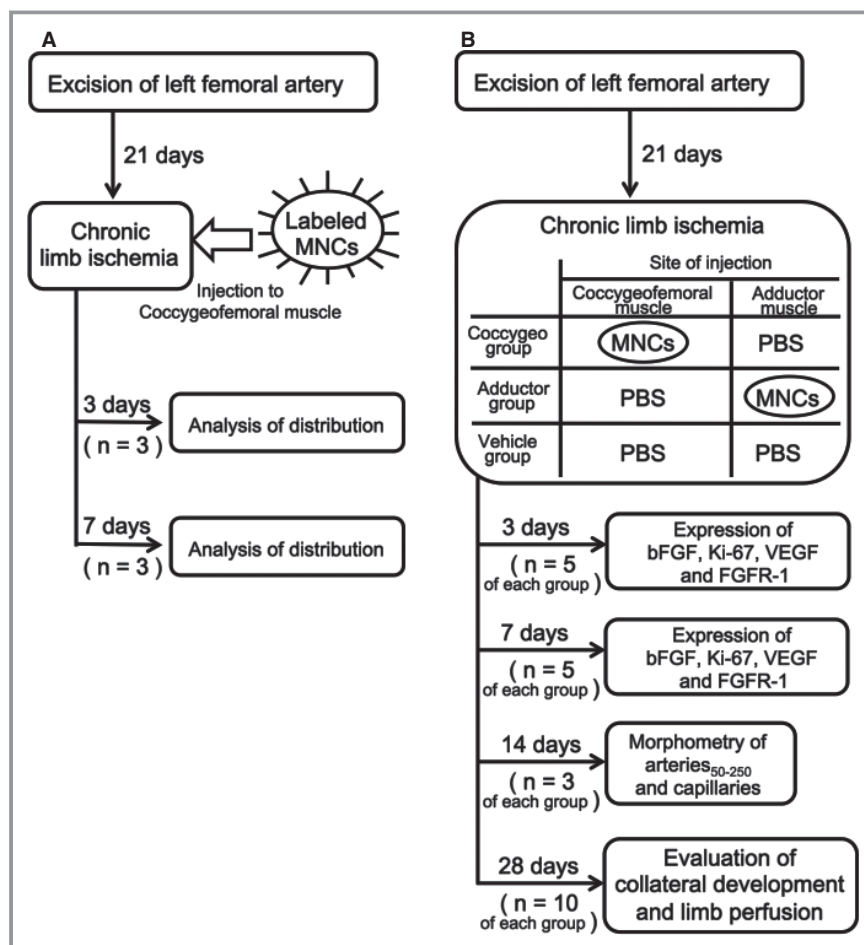


Figure 2. Experimental protocols. A, Distribution of bone marrow MNCs after injection in the coccygeofemoral muscle. B, Evaluations of collateral development and limb perfusion after the injection of MNCs. bFGF indicates basic fibroblast growth factor; FGFR-1, FGF receptor 1; MNCs, mononuclear cells; PBS, phosphate buffered saline; VEGF, vascular endothelial growth factor.

(5×10^6 cells) were harvested from the rabbits, as described. The number of MNCs was chosen based on previous studies in which MNCs were delivered for the treatment of an ischemic limb in the rabbit model.¹⁵ In the coccygeo group, the suspension of MNCs was injected at 3 different sites in the left coccygeofemoral muscle, as described, whereas a total volume of 1 mL PBS was injected at 3 different sites in the left adductor muscle. In the adductor group, the MNC suspension was injected at 3 different sites in the left adductor muscle, whereas a total volume of 1 mL PBS was injected at 3 different sites in the left coccygeofemoral muscle. In the negative control (vehicle) group, PBS was injected in both the left coccygeofemoral muscle and the adductor muscle. Calf blood pressure was measured in both hind limbs immediately after the injections, and calf blood pressure ratio—defined as the ratio of left systolic pressure to right systolic pressure—was calculated.^{1,16}

Evaluation of Collateral Vessel Development and Limb Perfusion

The development of collateral vessels in the coccygeo, adductor, and vehicle groups was evaluated at day 28 after injection of MNCs and PBS (MNCs/PBS) or PBS alone; this assessment period is the standard that was used in previous studies with the rabbit model.^{7,12,17} Initially, calf blood pressure was measured, and calf blood pressure ratio was calculated as described. Next, a 2.8-French end-hole catheter was inserted into the left iliac artery through the carotid artery, and a 0.036-cm (0.014-in) Doppler guidewire (Volcano) was introduced via the catheter into the proximal part of the left internal iliac artery. Average peak velocity was measured at rest, and then maximum average peak velocity was assessed after injection of 2 mg papaverine (Nichi-Iko Pharmaceutical Co). In vivo blood flow was calculated, as described previously.^{16,18} Next, the tip of the 2.8-French catheter was positioned at the level of the middle of the first sacral vertebra. Angiograms were taken, and angiographic scores were determined, as described previously.¹⁶

Analysis of the Coccygeofemoral Muscle During Collateral Development

To determine the mechanism of collateral vessel development after transplantation of MNCs, we conducted a histological investigation and evaluated protein expression in the coccygeofemoral muscle (Figure 2B). The rabbits in the coccygeo, adductor, and vehicle groups were sacrificed at days 3, 7, and 14 after the injection ($n=5$ in each group at days 3 and 7, $n=3$ in each group at day 14). Briefly, an infusion catheter was introduced into the abdominal aorta in the distal direction, and 200 mL of lactated Ringer's solution was infused at

120 mm Hg. In the rabbits sacrificed at days 3 and 7 after injection, the left coccygeofemoral muscle was excised and divided transversely into 8 parts with equal thickness. The fifth part from the muscle origin was embedded in an OCT compound (Miles) for immunohistological analysis, and the sixth part was snap-frozen in liquid nitrogen for protein extraction. These samples were stored at -80°C . The animals sacrificed at day 14 received an additional perfusion with phosphate-buffered 4% paraformaldehyde at 120 mm Hg, and the left coccygeofemoral muscle was divided into 8 parts in the same manner. The fifth part from the muscle origin was embedded in paraffin.

Morphometric Analysis of Collateral Vessels and Capillaries in the Coccygeofemoral Muscle

Transverse sections (4- μm thick) were cut from the paraffin-embedded muscle samples obtained from the animals sacrificed at day 14 after the injection; these transverse sections were used to analyze the densities of the developing collateral vessels and capillaries.^{7,12,17} The sections were stained with hematoxylin and eosin, and photomicrographs of each section were taken. The whole specimen area (specimen area) was measured for each section using ImageJ software (version 1.42q; National Institutes of Health), and the number of arteries with a diameter between 50 and 250 μm (arteries_{50–250}) was counted. The minimal value of several diameter measurements made in different directions was used as the artery diameter. Arteries_{50–250} correspond to developing collateral vessels because collateral vessels are generally developed by remodeling of small arteries with a diameter <50 μm .¹⁹ Density of arteries_{50–250} was calculated as follows:

$$\begin{aligned} \text{Arteries}_{50-250} \text{ density} (/ \text{mm}^2) \\ = \text{number of arteries}_{50-250} / \text{specimen area} \end{aligned}$$

Another set of sections was immunostained for CD31 to detect capillary density. After antigen retrieval (Dako), hydrogen peroxide treatment (Dako), and blocking with horse serum, a monoclonal antibody against CD31 (1:100 dilution; Dako) was applied and incubated for 60 minutes at 37°C . A biotinylated horse anti-mouse IgG antibody (Vector Laboratories) was subsequently applied and visualized using the ABC Elite Kit (Vector Laboratories). Photomicrographs were taken, and capillary density was calculated, as described previously.^{1,7,16}

Assessment of bFGF Expression With Western Blot

To assess the expression of bFGF in the coccygeofemoral muscle, frozen muscle samples (0.5 g) excised at days 3 and

7 after the injection were lysed in 1 mL of minimum essential medium (Invitrogen), and bFGF in each lysate was bound to heparin-Sepharose CL-6B (GE Healthcare). The heparin-Sepharose-bound samples were analyzed by Western blot using a monoclonal antibody against bFGF (Millipore) at a dilution of 1:500, as described previously.^{7,20}

Expression of Ki-67, VEGF, and FGF Receptor 1 in the Coccygeofemoral Muscle

Sections that were 4 μm thick and prepared from OTC-embedded muscle samples excised at days 3 and 7 after the injection were immunostained using a monoclonal antibody against Ki-67 (1:100 dilution; Dako), VEGF (1:50 dilution; Fitzgerald), or FGF receptor 1 (FGFR-1; 1:100 dilution; QED Bioscience). The expression of Ki-67 is associated with cell proliferation, and previous studies showed that the expression of Ki-67, VEGF, and FGFR-1 are markedly upregulated in and around collateral vessels during the early days after the induction of collateral development.^{12,21} After immunostaining, photomicrographs were taken, and the specimen area was measured on each section in the same manner. Numbers of Ki-67–positive cells, VEGF-positive cells, or FGFR-1–positive vessels were counted on each section, and the following parameters were calculated:

$$\begin{aligned} \text{Ki-67–positive cells density } (/mm^2) \\ = \text{Ki-67–positive cell number/specimen area} \end{aligned}$$

$$\begin{aligned} \text{VEGF-positive cells density } (/mm^2) \\ = \text{VEGF-positive cell number/specimen area} \end{aligned}$$

$$\begin{aligned} \text{FGFR-1–positive vessels density } (/mm^2) \\ = \text{FGFR-1–positive vessel number/specimen area} \end{aligned}$$

Statistical Analysis

Data are shown as mean \pm SE. Statistical significance was evaluated by the Tukey-Kramer method for multiple comparisons after the equality of data variances was confirmed using the Brown-Forsythe test. $P < 0.05$ was considered statistically significant.

Results

Distribution of MNCs After Injection in the Coccygeofemoral Muscle

Labeled MNCs were injected in the left coccygeofemoral muscle of the rabbits with chronic limb ischemia. At day 3 after injection, fluorescence microscopic images revealed that the labeled cells were abundantly distributed in the connec-

tive tissue between muscle fibers (Figure 3A). In contrast, no labeled cells were detected in the corresponding samples of the proximal adductor muscle (Figure 3B). Analysis of muscle samples obtained at day 7 revealed similar findings: The labeled cells were distributed in the coccygeofemoral muscle (Figure 3C) but not the adductor muscle (Figure 3D).

Evaluation of Collateral Vessel Development and Limb Perfusion

Development of collateral vessels and limb perfusion was evaluated at day 28 after the injection of MNCs/PBS or PBS alone. Although initially there were no differences in the calf blood pressure ratios between the 3 groups, the calf blood pressure ratio measured 28 days after the injection was significantly higher in the coccygeo group compared with the adductor and vehicle groups (Figure 4A). Interestingly, the calf blood pressure ratio in the adductor group was significantly higher than that in the vehicle group. The resting blood flow and maximum blood flow in the left internal iliac artery significantly improved in the coccygeo group compared with the adductor and vehicle groups (Figure 4B); however, there were no statistically significant differences in the resting blood flow and maximum blood flow values between the adductor and vehicle groups.

The angiography of the left internal iliac artery demonstrated marked development of collateral vessels in the coccygeo group and moderate collateral development in the adductor group (Figure 5A and 5B). In contrast, few collateral vessels were observed in the vehicle group (Figure 5C). The angiographic score was significantly higher in the coccygeo group than in the adductor and vehicle groups, and the score in the adductor group was significantly greater than in the vehicle group (Figure 5D).

Morphometric Analysis of Collateral Vessels and Capillaries in the Coccygeofemoral Muscle

The left coccygeofemoral muscle was resected from the rabbits at day 14 after the injection of MNCs/PBS or PBS alone. Hematoxylin and eosin–stained sections of the muscles revealed arteries with various sizes between the muscle fibers, and no damage to the muscle fibers was detected (Figure 6A through 6C). We focused on arteries_{50–250} (arteries with diameters between 50 and 250 μm) that corresponded to developing collateral vessels.¹⁹ In the coccygeo group, abundant arteries_{50–250} were observed between the muscle fibers, but few such arteries were observed in the adductor and vehicle groups (Figure 6A through 6C). In agreement with this finding, the density of arteries_{50–250} was significantly higher in the coccygeo group compared with the adductor and vehicle groups (Figure 6D), whereas there was no significant

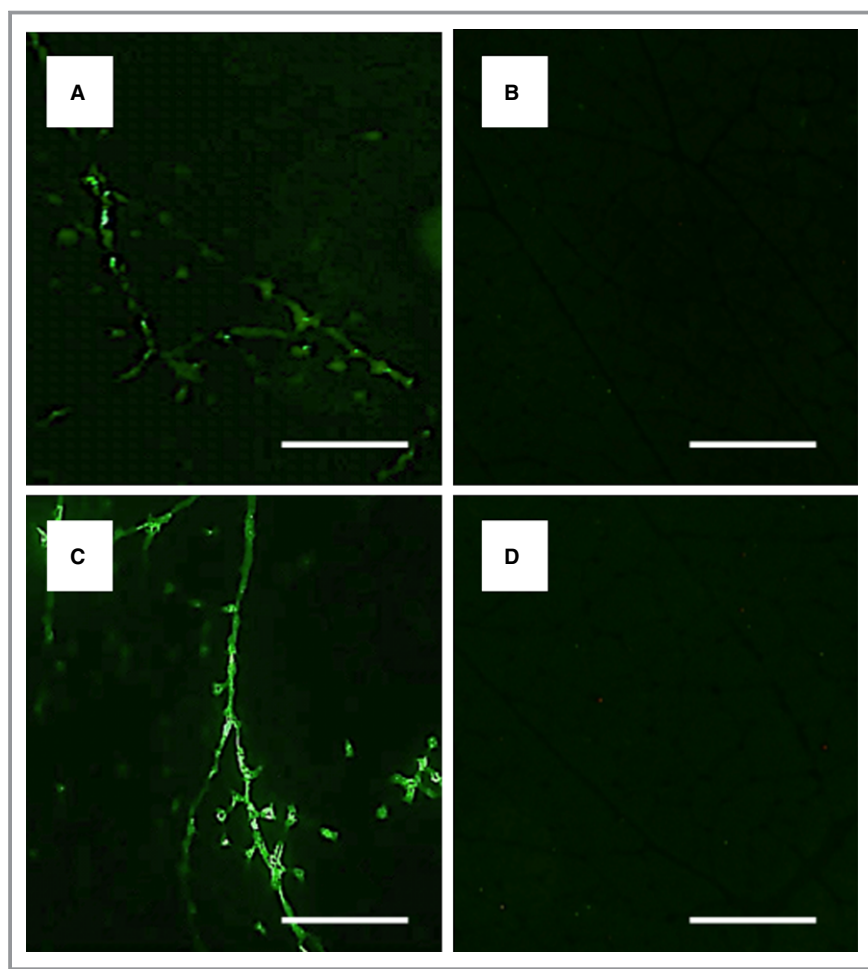


Figure 3. Fluorescence micrographs of the coccygeofemoral muscles after injection of bone marrow mononuclear cells (MNCs) labeled with green fluorescent marker. Upper panels correspond to day 3 after injection to the coccygeofemoral muscle (A) and the adductor muscle (B). Lower panels correspond to day 7 after injection to the coccygeofemoral muscle (C) and the adductor muscle (D). Labeled MNCs are colored green. Bar: 200 μ m.

difference between the values in the adductor and vehicle groups. Furthermore, analyses of the anti-CD31 immunostaining revealed that the capillary density in the coccygeo group was significantly greater than those in the adductor and vehicle groups (Figure 6E through 6H).

Expression of bFGF in the Coccygeofemoral Muscle After MNC Injection

Accumulation of bFGF in the left coccygeofemoral muscle at days 3 and 7 after the injection of MNCs/PBS or PBS alone was investigated by Western blot after bFGF concentration with heparin-Sepharose. Two forms of bFGF (18 and 22 kDa) were observed in some lysates of the coccygeofemoral muscle. In the coccygeo group, bFGF accumulation at day 3 was higher than in the adductor and vehicle groups, and abundant accumulation of bFGF continued at day 7 (Figure 7).

Moderate accumulation of bFGF was detected in the adductor group 7 days after injection.

Expression of Ki-67, VEGF, and FGFR-1 in the Coccygeofemoral Muscle After MNCs Injection

In immunostained samples of the coccygeofemoral muscle obtained at day 3 after injection of MNCs/PBS or PBS alone, Ki-67–positive cells, VEGF-positive cells, and FGFR-1–positive vessels were markedly increased in the case of the coccygeo group compared with the adductor and vehicle groups (Figure 8A through 8I). The Ki-67–positive cells and VEGF-positive cells were scattered in the connective tissue and vessel walls between muscle fibers. Quantitative analysis of the samples at day 3 also showed that the density of Ki-67–positive cells, VEGF-positive cells, and FGFR-1–positive vessels in the coccygeo group was significantly higher compared with the

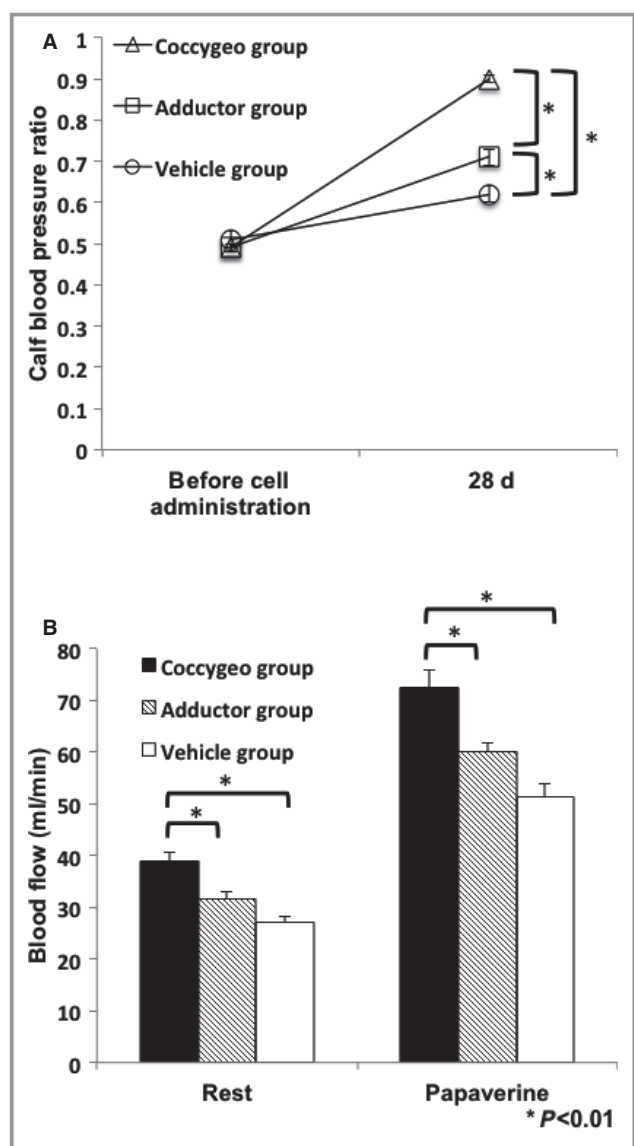


Figure 4. A, Calf blood pressure ratio immediately before and at 28 days after injection of mononuclear cells (MNCs) and PBS (MNCs/PBS) or PBS alone. B, Resting blood flow and maximum blood flow in the left internal iliac artery 28 days after injection of MNCs/PBS or PBS alone. PBS indicate phosphate buffered saline. * $P<0.01$.

adductor and vehicle groups (Figure 8J through 8L). Furthermore, at day 7 after injection, the density of Ki-67-positive cells and FGFR-1-positive vessels was significantly greater in the coccygeo group than in the adductor and vehicle groups, although no difference in the VEGF-positive cell density was detected among the 3 groups (Figure 8J through 8L).

Discussion

Collateral vessels generally develop from preexisting arteriolar connections that link different arterial trees at the peripheral

level. Depending on the location and extent of the arterial lesion, arteriolar connections spanning the lesion tend to form collateral vessels. The present study attempted to verify whether adequate delivery of autologous MNCs to arteriolar connections is important to promote effective collateral vessel development and limb perfusion in the rabbit model of chronic limb ischemia.

In our model, ischemia of the limb was initiated by complete excision of the femoral artery. This ischemia occurred mainly in the lower limb, and a previous study showed that collateral vessels tend to develop between the ipsilateral posterior gluteal artery and the branches of the popliteal artery.¹² The posterior gluteal artery is a branch of the internal iliac artery that distributes as a major feeding artery to the coccygeofemoral muscle from the proximal direction (Figure 1). The coccygeofemoral muscle also receives blood from minor feeding arteries branching off the popliteal artery, thus the periphery of the posterior gluteal artery is connected with that of the branches of the popliteal artery in the coccygeofemoral muscle. When blood flow through the femoral artery is completely shut off, an alternative route from the posterior gluteal artery to the popliteal artery via the arteriolar connections is used to supply the ischemic lower limb; however, because the amount of supplied blood is insufficient, the mechanism of arteriogenesis is induced in the arteriolar connections. Arteriogenesis, a form of angiogenic mechanism, refers to enlargement and maturation of preexisting arterioles.^{9–11,22,23} Induction of arteriogenesis in the arteriolar connections situated in the coccygeofemoral muscle leads to their enlargement and strengthening of the arterial walls and ultimately allows them to function as collateral vessels. Unfortunately, this enlargement of arteriolar connections is usually insufficient to compensate for the ischemia in the limb. Nevertheless, therapeutic stimulation of arteriogenesis in the arteriolar connections located in the coccygeofemoral muscle may result in the development of fully functional collateral vessels to the ischemic lower limb, thereby achieving the goal of therapeutic angiogenesis. It is notable that the coccygeofemoral muscle itself is not included in the ischemic area of this model.

In the present study, we used directed delivery of auto-MNCs to initiate therapeutic angiogenesis. Several studies have used MNCs in animal models of limb ischemia and reported favorable improvements of the ischemia, although the mechanism of the therapeutic effect has been controversial.^{4,15,24} An important role for endothelial progenitor cells present in the MNC fraction was initially suspected.^{13,25} Endothelial progenitor cells can differentiate and replicate to construct a new network of blood vessels in the process termed *vasculogenesis*, another form of angiogenic mechanism observed in vivo. It was expected that the newly formed

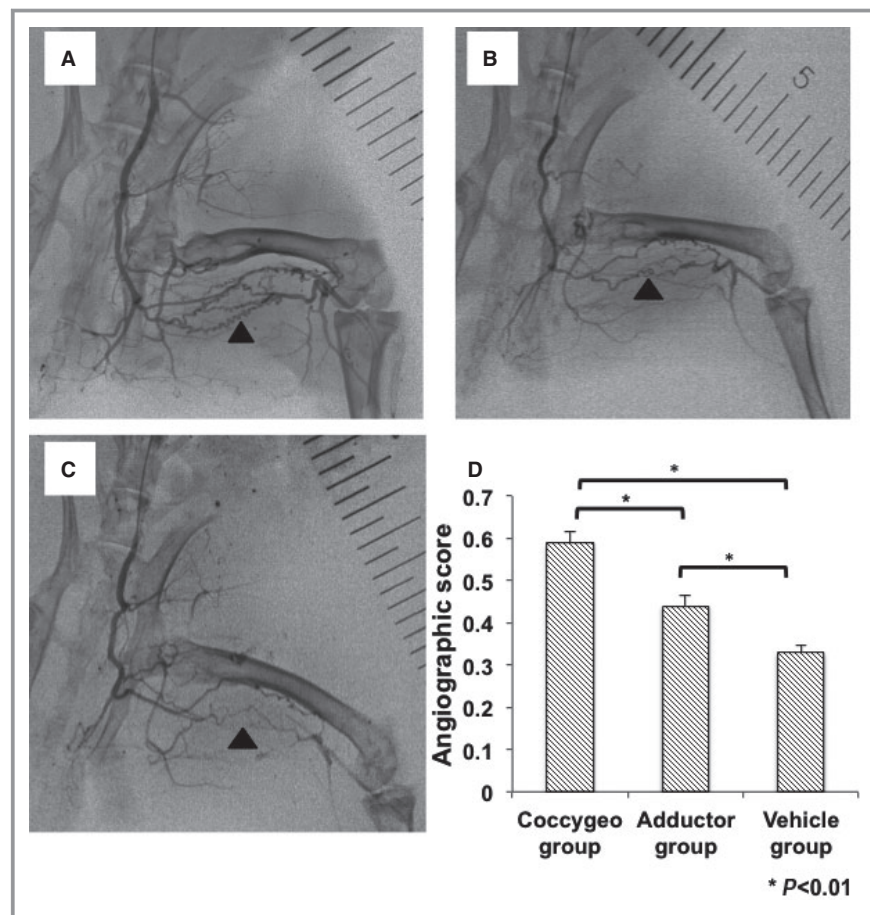


Figure 5. Selected internal iliac arteriograms at 28 days after injection of mononuclear cells and PBS or PBS alone. Arrowheads indicate collateral vessels. A, Coccygeo group. B, Adductor group. C, Vehicle group. D, Angiographic score. PBS indicate phosphate buffered saline. * $P < 0.01$.

vascular network could increase regional blood perfusion, thereby improving limb ischemia. Recent studies, however, revealed that transplantation of MNCs does not sufficiently stimulate vasculogenesis by endothelial progenitor cells and that improved blood perfusion in ischemic lesions is stimulated by bioactive factors released from MNCs, now considered the main mechanism of therapeutic angiogenesis with MNCs.^{26,27} Analysis of the bioactive factors released by MNCs identified a number of angiogenic growth factors, such as bFGF and VEGF, and inflammatory cytokines. Because these growth factors and cytokines can function to promote arteriogenesis, we hypothesized that delivery of MNCs may induce arteriogenesis in arteriolar connections.

Selective delivery of MNCs may be feasible only if the delivered cells are retained at the site of injection; therefore, we investigated the mobility of MNCs in the coccygeofemoral muscle by using fluorescently labeled cells. Histological analysis of muscle samples at days 3 and 7 after injection revealed that the labeled cells were distributed in the coccygeofemoral muscle, with no such cells detected in the

closely located adductor muscle; however, because the distribution analyses were based on histological findings from separate muscles samples, the results are neither quantitative nor comprehensive, and that is a limitation of this study. Nevertheless, these findings suggest that MNCs exhibit minimal migration into other muscles through the fascia, supporting the concept of selective delivery by intramuscular injection.

To investigate whether the coccygeofemoral muscle represents an adequate delivery site for effective collateral development and improvement of limb perfusion in our rabbit model, a sufficient number of auto-MNCs were delivered to the coccygeofemoral muscle or adductor muscle at day 21 after femoral artery removal. Although the adductor muscle is another major muscle in the thigh, its arteriolar connections have little potential to develop into collateral vessels in this model. At day 28 after injection, collateral circulation was evaluated in 3 different ways. Calf blood pressure ratio and blood flow in the left internal iliac artery, the conventional parameters used in many previous studies to assess collateral

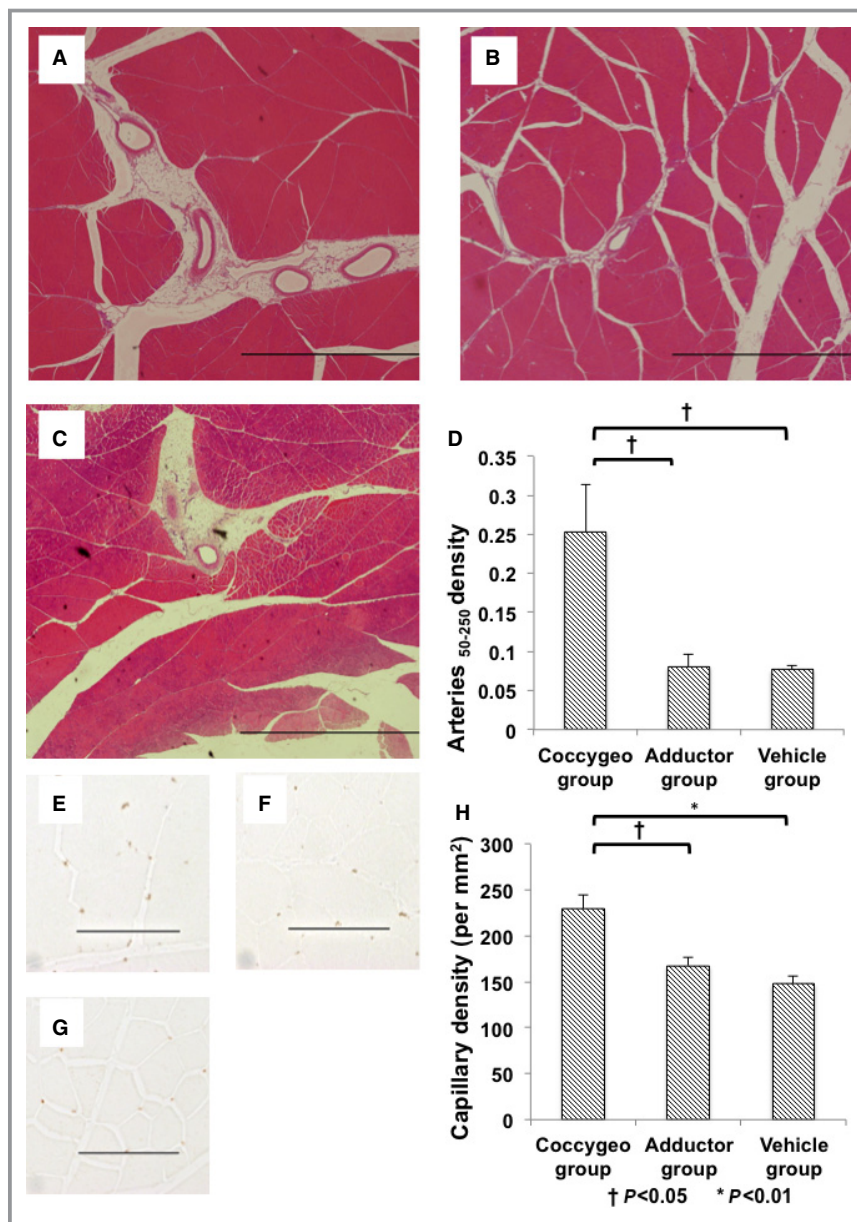


Figure 6. A through C, Hematoxylin and eosin–stained images of the coccygeofemoral muscle at day 14 after injection of mononuclear cells and PBS or PBS alone. A, Coccygeo group. B, Adductor group. C, Vehicle group. D, Density of arteries with diameters in the 50- to 250- μm range (arteries_{50–250}). Arteries_{50–250} correspond to developing collateral vessels. E through G, Immunostaining for CD31 in the coccygeofemoral muscle at 14 days after injection. E, Coccygeo group. F, Adductor group. G, Vehicle group. CD31-positive parts are colored brown. H, Capillary density. Bar: 200 μm . PBS indicate phosphate buffered saline. † $P < 0.05$.

perfusion in this model,^{12,17,28,29} were significantly higher in the coccygeo group than in the adductor and vehicle groups. This indicates that MNC delivery to the coccygeofemoral muscle is more efficient than delivery to the adductor muscle. Development of collateral vessels was also assessed using angiography. Because this method can visualize arteries with diameters $>50 \mu\text{m}$, functional vessels with mature wall structure are generally resolved, which allows estimation of

the degree of collateral development.³⁰ The angiographic scores in the coccygeo group were significantly higher than those in the adductor and vehicle groups. This confirms that the coccygeofemoral muscle is the preferable site for MNC delivery. Development of collateral vessels was also evaluated with morphometric analysis of the coccygeofemoral muscle at day 14 after the injection. Because diameters of most newly developed collateral vessels fall in the 50- to 250- μm range

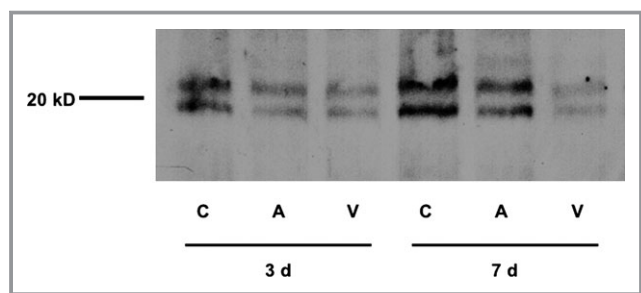


Figure 7. Basic fibroblast growth factor (bFGF) expression in the coccygeofemoral muscle at days 3 and 7 after injection of mononuclear cells and PBS or PBS alone. The bFGF was concentrated by binding to heparin-Sepharose and detected with Western blot. A indicates the adductor group; C, coccygeo group; PBS, phosphate buffered saline; V, vehicle group.

14 days after the induction of arteriogenesis, the higher density of arteries_{50–250} in the coccygeo group reflects more efficient development of collateral vessels compared with the adductor and vehicle groups and supports the results obtained with angiography. Taken together, these findings suggest that selective delivery of MNCs to the coccygeofemoral muscle initiated efficient development of collateral vessels and improvement of limb perfusion. Consequently, arteriolar connections in the coccygeofemoral muscle may represent an adequate therapeutic site in this rabbit model.

Schaper and coworkers demonstrated that intrinsic arteriogenesis is triggered by increased shear stress to the vascular lumen.^{8,19,23} This stimulates expression of monocyte chemoattractant protein 1 in vascular wall cells and adhesion molecules on the cell surface, leading to infiltration of inflammatory cells to the vessels. These inflammatory cells may release angiogenic growth factors, cytokines, and proteases, and these substances can cooperatively promote arteriogenesis. Importantly, MNCs can also release angiogenic growth factors and inflammatory cytokines, which may also induce arteriogenesis. Indeed, abundant accumulation of bFGF at days 3 and 7 after MNC delivery to the coccygeofemoral muscle was detected in the same muscle by Western blot. In addition, bFGF is thought to play an important role in the process of arteriogenesis.^{7,17} A reason for this is that bFGF possesses a broad spectrum of mitogenic activity for vascular wall cells. Not only does bFGF induce proliferation of endothelial cells, it also promotes replication of smooth muscle cells in media and fibroblasts in the adventitia. Because arteriogenesis involves growth of all components of vascular wall cells, the broad mitogenic activity of bFGF might effectively contribute to this process. To assess cell proliferation after the delivery of MNCs, we evaluated the expression level of Ki-67 in the coccygeofemoral muscle. The number of Ki-67–positive cells increased in and around the vessels after the delivery, which

coincided with bFGF accumulation. In addition, bFGF is important because of its potent angiogenic effects through the paracrine mechanism. Previous studies reported that bFGF could stimulate several stromal cells to release other angiogenic growth factors, such as VEGF and hepatocyte growth factors.^{31–33} In this regard, VEGF-positive cells in the coccygeofemoral muscle markedly increased after the delivery of MNCs, although VEGF expression could also be induced by other bioactive substances released from MNCs. Of note, bFGF and VEGF were shown to exhibit a synergistic effect in arteriogenesis.¹⁷ Furthermore, it is known that during arteriogenesis, vascular wall cells upregulate the expression of FGFR-1, a specific receptor of bFGF. In this study, increased expression of FGFR-1 was observed in the vessels of the coccygeofemoral muscle after the delivery of MNCs. Upregulated FGFR-1 might further enhance the effects of released bFGF. Meanwhile, bFGF and VEGF are potent promoters of capillary formation, and we observed that capillary densities significantly increased in the coccygeofemoral muscle after the delivery of MNCs; however, it is unclear whether capillary augmentation in the coccygeofemoral muscle contributed to the improvement of limb ischemia.

An interesting finding of the present study is that the calf blood pressure ratios and angiographic scores in the adductor group showed significant improvement compared with those in the vehicle group, although the values in the adductor group were still lower compared with those in the coccygeo group. This finding was unexpected because arteriolar connections in the adductor muscle possess little potential for collateral vessel development. Migration of angiogenic substances from the adductor muscle to the coccygeofemoral muscle is the most plausible explanation. Although we suspect that MNCs that were injected into the adductor muscle remained in the same muscle, angiogenic factors that they release may be able to migrate to the arteriolar connections in the coccygeofemoral muscle. In agreement with this hypothesis, Western blot analysis of the coccygeofemoral muscle showed moderately intense bands of bFGF at day 7 after the delivery of MNCs to the adductor muscle.

In summary, we demonstrated that selective delivery of autologous bone marrow MNCs to the coccygeofemoral muscle in the rabbit model of chronic limb ischemia markedly enhanced collateral vessel development and limb perfusion at day 28, whereas delivery to the adductor muscle did not have such an effect. These findings indicate that adequate selection of the delivery site is important to achieve effective augmentation of collateral vessels in therapeutic angiogenesis with MNCs. Furthermore, arteriolar connections that are likely to develop into collateral vessels might constitute an adequate delivery site. We consider that the results of this study are applicable to clinical therapeutic angiogenesis using MNCs.³⁴ If an appropriate therapeutic site could be identified

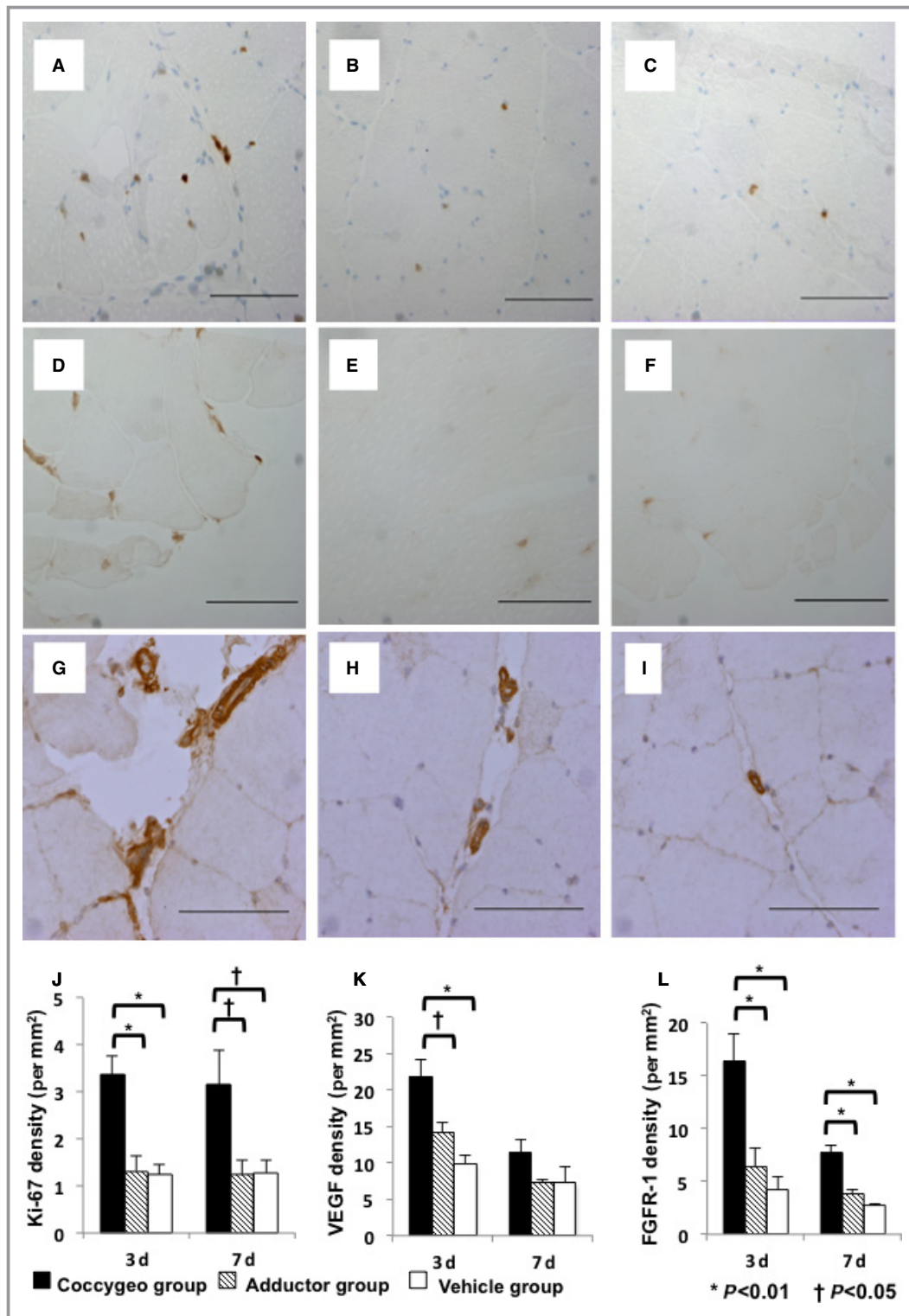


Figure 8. Immunostaining images of the coccygeofemoral muscle at day 3 after injection of mononuclear cells and PBS or PBS alone. The sections of the coccygeofemoral muscle were stained for Ki-67 (A through C), vascular endothelial growth factor (VEGF) (D through F), or fibroblast growth factor receptor 1 (FGFR-1) (G through I). Panels A, D, and G represent samples from the coccygeo group; panels B, E, and H represent samples from the adductor group; and panels C, F, and I represent samples from the vehicle group. Antigen-positive parts are colored brown. Bar: 100 μ m. To quantify immunostaining of samples at days 3 and 7, Ki-67–positive cell density (J), VEGF–positive cell density (K), and FGFR-1–positive vessel density (L) were measured. PBS indicate phosphate buffered saline. * $P < 0.01$, † $P < 0.05$.

in human patients, selective delivery of MNCs to that site might facilitate more effective therapeutic angiogenesis in these patients; however, the pattern of collateral development in humans differs from that in rabbits, and arterial lesions in humans are generally more diverse and complex than those in the rabbit model. Future studies regarding the human pathophysiology of collateral development are likely needed to precisely identify an adequate therapeutic site in humans.

Disclosures

None.

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