Combination of In Vivo Angiopoietin-1 Gene Transfer and Autologous Bone Marrow Cell Implantation for Functional Therapeutic Angiogenesis

Koichi Kobayashi, Takahisa Kondo, Natsuo Inoue, Mika Aoki, Masaaki Mizuno, Kimihiro Komori, Jun Yoshida, Toyoaki Murohara

Objective—Autologous bone marrow mononuclear cell (BM-MNC) implantation into ischemic tissues promotes angiogenesis, but a large amount of marrow aspiration is required, which is a major clinical limitation. Angiopoietin-1 (Ang-1) is requisite for vascular maturation during angiogenesis. We examined the impacts of combinatorial Ang-1 gene transfer and low-dose autologous BM-MNC implantation on therapeutic angiogenesis in a rabbit model of hind limb ischemia.

Methods and Results—Rabbits were divided into 4 groups: phosphate-buffered saline (control), 500 μg Ang-1 plasmid (Ang-1), $1 \times 10^6$ autologous BM-MNCs (BMC), and Ang-1 plasmid plus BM-MNCs (combination). The Ang-1 group had a greater angiographic score and capillary density compared with the control ($P<0.05$), but the Ang-1 gene therapy alone did not improve transcutaneous oxygen pressure (TcO$_2$) and skin ulcer score. However, the combination group showed a significant improvement in not only angiographic score and capillary density ($P<0.05$) but also TcO$_2$ ($P<0.05$) and skin ulcer score. These efficacies were greater in the combination group compared with the BMC group.

Conclusions—This Ang-1 gene and BM-MNC combination therapy enhances not only quantitative but also qualitative angiogenesis in ischemic tissues. Moreover, the combination therapy will enable a reduction in the amount of BM aspiration required for significant therapeutic angiogenesis. (Arterioscler Thromb Vasc Biol. 2006;26:1465-1472.)

Key Words: angiogenesis • angiopoietin-1 • bone marrow cells • vasculogenesis

The objective of therapeutic angiogenesis is to treat no-option patients with severe ischemic heart disease and peripheral artery disease. Sole therapy with angiogenic cytokines, such as vascular endothelial growth factor (VEGF) or basic fibroblast growth factor (bFGF), enhanced collateral circulation in patients with peripheral artery disease. However, initial enthusiasm has been tempered by a series of negative results in randomized clinical trials. Precise reasons for the ineffectiveness of therapeutic angiogenesis using a sole growth factor in clinical trials have not been well elucidated. However, Ozawa et al reported that one of the key determinants of successful angiogenesis was the amount of growth factors within tissues. Growth factor contents in tissues beyond angiogenic threshold levels created rather pathological vessels. We then reasoned that both normal structural development and an absolute quantity of vessels are required for the successful and functional outcome of therapeutic angiogenesis.

Implantation of endothelial progenitor cells (EPCs) or bone marrow (BM) mononuclear cells (MNCs) has been shown to augment neovascularization of ischemic tissues by supplying EPCs into vasculature and by secretion of various angiogenic growth factors. These results suggest that implantation of autologous BM-MNCs induces functional angiogenesis, but the limited number or amount of EPCs or BM-MNCs in patients with coronary and peripheral artery disease may offset their overall therapeutic efficacy.

Angiopoietin-1 (Ang-1) has been identified as a ligand for a receptor tyrosine kinase Tie-2 expressed on endothelial cells and hematopoietic stem cells. Ang-1 has little effect on proliferation but induces migration, sprouting, chemotaxis, survival, and network formation of endothelial cells. Moreover, Ang-1 is requisite for vascular maturation and stabilization via endothelial attachment to extracellular matrices. Gene transfer of Ang-1 expression plasmid indeed significantly enhanced neovascularization in vivo.

Accordingly, we investigated whether the combination therapy with in vivo Ang-1 plasmid gene transfer and autologous BM-MNCs implantation would promote functional neovascularization in a rabbit model of operatively induced unilateral hind limb ischemia.
Materials and Methods

Rabbit BM-MNCs Isolation
All animal protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Rabbit BM was aspirated from the right iliac crest. BM-MNCs were isolated by centrifugation through a Histopaque density gradient (Sigma, St. Louis, Mo) as described previously.8,28

Rabbit Model of Unilateral Hind Limb Ischemia
Neovascular formation was examined using a rabbit model of unilateral hind limb ischemia.8,29 The femoral artery of male New Zealand White rabbit was excised from its proximal origin to the distal point where it bifurcates into the saphenous and popliteal arteries.

Therapeutic Neovascularization With Angiopoietin-1 Plasmid and/or Autologous BM-MNCs
Rabbits (n=40) were subjected to unilateral hind limb ischemia and randomly divided into 4 groups. On day 7 after the induction of limb ischemia, systolic calf blood pressure measured by a sphygmomanometer (BP-98E; Softron, Tokyo) in ischemic limb was <40 mm Hg in all rabbits, and there was no difference among the experimental groups. Rabbits received empty plasmid vector (pCA1) plus phosphate-buffered saline injection (control group, n=10), Ang-1 plasmid (pCAhAng-1) plus phosphate-buffered saline injection (Ang-1 group, n=11), pCA1 plus BM-MNC transplantation (BM-MNC group, n=10), pCAhAng-1 plus BM-MNC transplantation (combination group, n=9). In brief, 100 μg of empty plasmid (pCA1) or Ang-1 plasmid (pCAhAng-1) was injected at 5 sites (total, 500 μg/2.5 mL) on day 7 after the induction of limb ischemia. On day 10, autologous BM-MNCs (1×10^6) were transplanted at 5 sites in thigh muscles.

Gene Expression in the Rabbit Muscles After Hind Limb Ischemia
Using reverse-transcription polymerase chain reaction (PCR) analysis, we examined the expression of plasmid-specific Ang-1 mRNA in the rabbit muscles. Human-specific Ang-1 mRNA was detected using a specific primer set overlapping the human Ang-1 gene and the rabbit β-globin terminator of the pCAac vector.30 Furthermore, the efficiency of transgene expression was evaluated morphometrically with the use of a promotor-matched reporter plasmid, pCAβ, that encodes β-galactosidase.

Iliac Angiography
Formation of collateral vessels was evaluated by iliac angiography on postoperative day 35.8 We calculated the angiographic score as described previously.8,29 Angiographic luminal diameter of the internal iliac artery in the ischemic hind limb was measured. The time period that the contrast medium flowed from the internal iliac artery to either the saphenous or popliteal arteries (contrast filling time) was also measured.

Doppler Guide Wire Measurement
Doppler flow wire (FloWire; Cardiometrics, Mountain View, Calif) was advanced to the internal iliac artery supplying the ischemic limb. Acetylcholine (1.5 μg/kg per minute) and nitroglycerin (50 μg/kg) were respectively administered to assess vasomotor reactivity.31,32 To evaluate endothelial function of newly formed vessels in the ischemic limb, the ratio of the endothelium-dependent flow increase (acetylcholine) to the endothelium-independent flow increase (nitroglycerin) was calculated.

Transcutaneous Oxygen Pressure Measurements
Transcutaneous oxygen pressure (TcO2) was measured (TCM-400; Radiometer, Copenhagen, Denmark). Measurements were performed on the dorsum, crus, and thigh of both hind limbs on day 35 and the average TcO2 value of the 3 skin points was calculated. The ischemic/normal hind limb TcO2 ratio was calculated.

Capillary-to-Muscle Fiber Ratio
Tissue specimens were obtained from the adductor and semimembranous skeletal muscles on day 35. Frozen tissue sections with 5-μm thickness were prepared. Alkaline phosphatase-positive capillary endothelial cells were counted under light microscopy (>200). Five fields from each sample were randomly selected for the counts. To ensure that the capillary density was not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary-to-muscle fiber ratio was also determined.

Evaluation of Ischemic Skin Ulcer of Hind Limb
On postoperative day 35, we evaluated the extent of skin necrosis/ulcer and limb loss in the ischemic hind limbs and classified the magnitude as follows: grade 0, no skin ulcer; grade 1, ulcer <2 cm in longer diameter; grade 2, intermediary between grades 2 and 3; grade 3, ulcer expanding a half of crus in the major axis; and grade 4, auto-amputation of distal lower limb with bony exposure.

In Vitro Assay for EPC Function
EPC Migration Assay
BM-MNCs isolated from additional rabbits were cultured in Medium-199 supplemented with 20% fetal bovine serum, bovine pituitary extract, heparin, and antibiotics. At day 7 of culture, adherent spindle-shaped cells were used in EPC migration assay.8,28 EPC migration in response to rhAng-1 and/or rhVEGF-A (R&D Systems, Minneapolis, Minn) was analyzed using a modified Boyden chamber apparatus (Neuroprobe, Gaithersburg, Md).28 Data were expressed as the number of migrated cells/field from the microvascular endothelial cells. EPC-like adherent cells were stained with an anti-Tie-2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and secondary antibody with Alexa Fluor 488 (Molecular Probes).

Endothelial Network Formation in Basement Matrix Gel
BM-MNCs were cultured in Medium-199 supplemented with 20% fetal bovine serum and different growth factor conditions (control medium, rhAng-1 50 ng/mL, or rhVEGF 50 ng/mL). On day 10 of BM-MNCs culture, spindle-shaped adherent cells (6×10^5) were labeled with a green fluorescent dye PKH2-GL (Sigma) and cocultured with unlabeled human umbilical vein endothelial cells (HUVECs) (6×10^5) on growth factor reduced Matrigel (Becton-Dickinson). Matrigel was incubated at 37°C for 12 hours.33 Number of labeled EPCs incorporated into the HUVEC tube formation was assessed in 3 random high-power fields (>100) per group.

Ex Vivo Whole Mounts of the Mouse Ear Vasculature
rhVEGF (150 ng), rhAng-1 (150 ng), and littersmate BM cells (1.5×10^6) were injected into the posterior auricular muscle of male C57BL/6J mice (6 to 8 weeks old). Recombinant protein was injected daily for 3 days, and BM cells of littermate were implanted on the first day. The entire vascular network of the ear was visualized after intravascular vital staining with a fluorescein-labeled Lectococcus esculentum lectin (Vector Laboratories, Burlingame, Calif) that binds the luminal surface of all blood vessels.6,23,34 Whole mounts of the ear skin were analyzed using a confocal laser scanning microscope (MRC-1024; BIO-RAD, San Diego, Calif) and photographed.

Materials and Methods are described online in detail (please see http://atvb.ahajournals.org).
the Kruskal-Wallis test. Correlation coefficients were calculated using Spearman rank correlation. Statistical significance was assumed at \( P < 0.05 \); \( n \) represents the number of animals.

**Results**

**Expression of Therapeutic Gene**

In preliminary experiments, Ang-1 transgene expression was evaluated at the mRNA level with reverse-transcription PCR in rabbits with hind limb ischemia at 3 days after gene therapy. To ensure specificity and to avoid amplification of endogenous rabbit Ang-1 mRNA, PCR primer set was selected from a region that is not conserved between the 2 species (please see http://atvb.ahajournals.org). The size of the PCR product for human-specific Ang-1 was 453 bp. PCR products were analyzed with 2% agarose gel electrophoresis, which showed a clear expression of human Ang-1 mRNA in gene-injected skeletal muscles but not in the control nontreated legs.

In ischemic limb muscles transfected with pCA, evidence of successful transfection, indicated by dark-blue staining with X-gal solution, was observed.

**Combination of Ang-1 Gene and BM-MNCs Therapy Increases Number of Large and Small Collateral Vessels and Diameter of Internal Iliac Artery**

On postoperative day 35, all animals were subjected to iliac arteriography. Representative angiograms are shown in Figure 1A. Collateral vessels as assessed by the angiographic score increased in all the treatment groups compared with the control group. However, tortuous collateral vessels with pathological corkscrew-like appearance developed only in the Ang-1 gene plasmid (Ang-1) group (Figure 1A). Quantitative analyses showed a greater angiographic score in the Ang-1 group and the Ang-1 gene plus BM-MNCs (combination) group compared with the control group (\( P < 0.05 \)) (Figure 1B). The BM-MNCs (BMC) group tended to have a greater but not statistically significant angiographic score. Luminal diameter of the internal iliac artery in the ischemic limb was significantly greater only in the combination group compared with the control group (\( P < 0.05 \)) (Figure 1C).

**Combination of Ang-1 Gene and BM-MNCs Therapy Increases the Capillary-to-Muscle Fiber Ratio**

The capillary/muscle fiber ratio was calculated as specific evidence of vascularization at the microvascular level. Representative photomicrographs of histological sections stained with alkaline phosphatase in the ischemic tissues are shown in Figure 2A. Histological examination revealed the presence of numerous capillary endothelial cells in ischemic skeletal muscle tissues in the 3 treatment groups compared with the control group. Quantitative analyses showed that the capillary/muscle fiber ratio was significantly greater in the 3 treatment groups compared with the control group (Figure 2B). The combination group showed the greatest capillary-to-muscle fiber ratio among the 3 treatment groups.

**Combination of Ang-1 Gene and BM-MNCs Therapy Promotes Functional Neovascularization**

To examine the functional aspects of the developed collateral circulation and angiogenesis, resting baseline blood flow, acetylcholine (Ach)-stimulated blood flow, and nitroglycerin-stimulated blood flows were measured at the level of the internal iliac artery using a Doppler flow wire on postoperative day 35. We measured the ratio of the endothelium-dependent (Ach)/endothelium-independent (nitroglycerin) flow response to evaluate endothelial function. The ratio was greater in the combination group than in the other 3 groups. Thus, combination therapy tended to enhance endothelial function in collateral circulation.

We also measured filling time of the contrast medium between the internal iliac artery and the bifurcation point of the femoral artery as an index of collateral blood flow velocity. The filling time was significantly shorter in the combination group than in the control and the Ang-1 group. These results indicated that collateral vessels were most
effectively functioning in the combination group compared with the other groups.

Combination Therapy but not Ang-1 Gene Sole Therapy Enhances Tissue Oxygenation in Ischemic Hind Limb

We evaluated tissue oxygenation using transcutaneous oxygen pressure (TcO2) measurement, as an index of functionality of neovascularization, on postoperative day 35. The ratio of the mean TcO2 of the ischemic/nonischemic hind limb (the ischemic/normal TcO2 ratio) was significantly greater in the combination group than in either the control or Ang-1 group (Figure 3A), indicating a marked improvement of microcirculation in the combination groups compared with the control group. Therefore, despite significant increases in the angiographic score and capillary density, the TcO2 ratio was not improved in the Ang-1 gene sole therapy group.

Combination Therapy but not Ang-1 Gene Therapy Reduces Ischemic Skin Ulcer and/or Limb Necrosis

In contrast to the anatomic and quantitative indices of angiogenesis (ie, angiographic score and capillary density), there were marked differences in the skin ulcer score among the 4 experimental groups. We observed extensive skin ulcer and auto-amputation of distal lower limb only in the control and Ang-1 groups (Figure 3B). In the Ang-1 group, quantitative angiogenic indices such as angiographic score and capillary density were significantly increased; however, functional indices such as TcO2 and skin ulcer score were not improved compared with the control group. In contrast, the combination therapy most improved the skin ulcer score among the experimental groups. These data show a clear inverse correlation between the skin ulcer score and the ischemic/normal hind limb TcO2 ratio (P=0.08 and ρ=0.34, Spearman rank correlation).

Recombinant Human Ang-1 Protein Facilitates EPCs Migration In Vitro

Next, we explored potential mechanisms by which Ang-1 gene therapy enhanced therapeutic angiogenesis induced by BM-MNC implantation. First, we stained ex vivo expanded BM-derived EPC-like attaching cells with an anti-Tie-2 antibody. A large part of the surface of attaching cells was stained positive with the anti-Tie-2 antibody (Figure 4A), indicating a robust expression of Tie-2 on the surface of EPCs. Using a modified Boyden chamber apparatus, we assessed the migratory response of ex vivo expanded BM-derived EPC-like attaching cells toward recombinant human
Ang-1 (rhAng-1) (25, 50, 100 ng/mL). We also compared the effects with those induced by rhVEGF (50 ng/mL), which is known to induce migration of EPCs.35 Both rhAng-1 and rhVEGF stimulated the migration of EPCs compared with the nontreated control group (Figure 4B). Moreover, the maximum migratory effects of rhAng-1 attained with 50 ng/mL seemed to be almost equivalent to those of rhVEGF (Figure 4C).

**rhAng-1 Promotes EPC Incorporation Into Endothelial Tube Formation**

To elucidate the effects of rhAng-1 on the differentiation capacity of BM-derived EPC-like attaching cells, we quantified the incorporation rate of cultured EPCs into tubular networks formed by HUVECs on Matrigel. rhAng-1 and/or rhVEGF were administered into the medium for EPC differentiation from BM-MNCs but not in the culture medium for the tube formation assay. EPCs cultured in medium containing both rhVEGF (10 ng/mL) and rhAng-1 (50 ng/mL) were markedly incorporated into tubular networks of HUVECs compared with EPCs cultured in rhVEGF (10 ng/mL) alone (Figure 5).

**Implantation of Bone Marrow Cells Prevents Formation of Pathological Vessels Induced by Excessive rhAng-1 in Mouse Ear**

rhAng-1 gene sole therapy failed to rescue limb amputation and did not increase ischemic/normal TcO2 ratio, although rhAng-1 increased the angiographic score and capillary/muscle fiber ratio. We speculated that Ang-1 mediated neovascularization was little functioning despite increased quantity. Therefore, we finally examined microvascular structures using an intravital mouse ear angiogram to estimate morphology of vessels formed by rhAng-1 and/or BM cells. We examined morphology of blood vessels in whole mounts of the mouse ears after staining with an intravenous injection of fluorescein isothiocyanate (FITC)-lectin (Figure 6A). The number of vessels in the ears receiving BM cells increased to some extent without morphological derangement. In contrast, injection of rhAng-1 (150 ng/d for 3 days) alone into the ear skin caused growth of abnormal corkscrew-like vessels with irregular lumens. Some vessels were dilated and formed microaneurysm and hemangiomas. Next, we examined whether BM cell implantation would ameliorate the pathological structures induced by rhAng-1. As shown in Figure 6A, the ears receiving BM cells in combination with rhAng-1 developed abundant capillary vessels without deleterious pathological vascular structures. Moreover, we evaluated the numbers of hemangioma or microaneurysm in 5 microscopic fields using additional mice (Figure 6B). These results indicate that rhAng-1 alone induced neovascularization but also formed abnormal vascular structures, and that BM cell implantation cancelled the formation of abnormal vascular structures by rhAng-1 and stimulated neovascularization further.
protein (eg, VEGF or bFGF) failed to improve clinical skin ulcer score and deterred autoamputation. Functional angiogenesis accompanied by a reduced ischemic MNCs and Ang-1 gene therapy cooperatively promoted quantitative angiogenic indices. The combination of BM-
failed to induce functional angiogenesis albeit it increased findings suggest that single Ang-1 gene or protein therapy vascular structures in the mouse ear vessel model. Our nation with rhAng-1 significantly enhanced angiogenesis in ear vessel analysis; and (5) BM cell implantation in combi-
combination therapy; (4) rhAng-1 alone induced pathological angiogenesis such as corkscrew-like vessels with irregular lumens, microaneurysm, and hemangioma-like structures. Combination of rhAng-1 and BM cells increased vascularity with only few pathological structures. B, The formation of aberrant vessels like hemangioma or microaneurysm was suppressed in the combination group compared with the rhAng-1 group. Similar results were obtained in 2 additional experiments. Bars=100 μm.

Figure 6. A, Whole mount of mouse ear vasculature stained with intravenous FITC-lectin. BM cell implantation increased the vascularity without any pathological morphology changes. However, rhAng-1 injection induced pathological angiogenesis with corkscrew-like vessels with irregular lumens, microaneurysm and hemangioma-like structures. Combination of rhAng-1 and BM-MNCs prevented the aberrant vascular structures in-
vented the formation of pathological vessels induced by rhAng-1. BM-MNCs were disorganized, leaky, poorly perfused, and tortuous, much like those observed in tumor vessels.

In the present study, Ang-1 sole therapy induced the formation of pathological vessels with irregular lumens. Previous studies have reported that Ang-1 induces nonleaky vascular formation through the vessel stabilization. But their studies did not demonstrate the whole structure of microvessels. Suri and et al reported that subcutaneously Ang-1 overexpressed transgenic mice have markedly dilated and pathologically branched subcutaneous microvessels. Their study may explain potential reasons why Ang-1 alone induced corkscrew like vessel formation in the present study.

In a rabbit model, we stained vascular smooth muscle cells in histological sections with an anti-SM1 (isoform of smooth muscle myosin heavy chain) antibody. There was an increase in the absolute number of vascular smooth muscle cells in the Ang-1–treated group; however, there was no significant difference in the ratio of SM1 positive vessels to the total vessels among the 4 experimental groups (data not shown). We consider that Ang-1 gene therapy facilitated mural cell recruitment but this salutary effect was overwhelmed with pathological vessel formation because of strong sprouting effects of Ang-1 on endothelial cells.

However, implantation of autologous BM-MNCs prevented the formation of pathological vessels induced by rhAng-1 in the mouse ear vessel model. This result indicates that combined administration of BM-MNCs raised the threshold for induction of aberrant vessels by rhAng-1. BM-MNCs might have secreted various angiogenic cytokines that harmonized the process of natural neovascularization together with Ang-1. It would be hypothesized that transplanted BM-MNCs prevented the aberrant vascular structures induced by Ang-1 sole gene therapy, resulting in the improvement of skin ulcer score and TcO2 in the rabbit hind limb model. To our knowledge, this is the first report to have clearly demonstrated that the combination of gene therapy and small amount of autologous BM cell implantation significantly enhances “functional” therapeutic neovascularization.

Because Ang-1 has a direct action on endothelial cells to facilitate migration and to inhibit apoptosis, we examined whether rhAng-1 has promoting action on migration and tube formation of EPC-like attaching cells expanded from rabbit BM-MNCs. Interestingly, rhAng-1 significantly enhanced migration of EPCs in vitro. Moreover, BM-MNCs pre-cultured in the presence of rhAng-1 were more abundantly

Discussion

The major findings of the present study are: (1) the Ang-1 gene sole therapy failed to improve skin ulcer score and tissue oxygenation (TcO2), 2 functional parameters, in ischemic limbs, although it enhanced the angiographic score and histological capillary density, 2 anatomic parameters; (2) the Ang-1 gene and BM-MNCs combination therapy significantly augmented the angiographic score and capillary density, accompanied by functional improvement of skin ulcer score and TcO2 in the ischemic limb; (3) the BM-MNC implantation sole therapy increased capillary density and TcO2, but to a lesser degree than those induced by the combination therapy; (4) rhAng-1 alone induced pathological neovascularization such as corkscrew-like vessels with irregular lumens, microaneurysm, and hemangioma in the mouse ear vessel analysis; and (5) BM cell implantation in combination with rhAng-1 significantly enhanced angiogenesis in both rabbit ischemic hind limb without inducing pathological vascular structures in the mouse ear vessel model. Our findings suggest that single Ang-1 gene or protein therapy failed to induce functional angiogenesis albeit it increased quantitative angiogenic indices. The combination of BM-MNCs and Ang-1 gene therapy cooperatively promoted functional angiogenesis accompanied by a reduced ischemic skin ulcer score and deterred autoamputation.

Several recent clinical trials using sole angiogenic gene or protein (eg, VEGF or bFGF) failed to improve clinical outcomes compared with placebo control in patients with critically ischemic diseases. In the angiogenic process, a harmonious interplay of various growth factors, cytokines, vasoactive substances, and cells of the vascular and inflammatory components may be critical to ensure the promotion of functional vessels. Therefore, it seems that administration of a single growth factor may not be sufficient to orchestrate such a complex cascade to form a truly functioning vascular network. This phenomenon is often called “delivery of too much of a good thing,” which leads to the formation of disorganized vessels rather than functional vessels. For example, vessels that developed in the cardiac and skeletal muscles after transplantation of VEGF-transfected myoblasts were disorganized, leaky, poorly perfused, and tortuous, much like those observed in tumor vessels.

Although a complete understanding of the complex interaction between Ang-1 and its receptors in the angiogenesis remains unknown, we know that Ang-1 has a direct action on endothelial cells to facilitate migration and to inhibit apoptosis. Whether rhAng-1 has promoting action on migration and tube formation of EPC-like attaching cells expanded from rabbit BM-MNCs remains to be elucidated. Interestingly, rhAng-1 significantly enhanced migration of EPCs in vitro. Moreover, BM-MNCs pre-cultured in the presence of rhAng-1 were more abundantly
incorporated into HUVEC tube formation compared with nontreated cells. The latter finding is supported by the fact that the Ang-1-mediated pathway is involved in the differentiation of endothelial cells from EPCs.45 Taken together, combined Ang-1 gene therapy might have further enhanced angiogenic actions of BM-MNCs after implantation by stimulating migration and differentiation of progenitor cell into endothelial lineage.

Another interesting finding is that Ang-1 and BM-MNCs combination therapy improved endothelial function (endothelium-dependent relaxation) of stem collateral vessel (the internal iliac artery) as assessed by the ratio of Ach-induced/nitroglycerin-induced vasodilation. The combination of Ang-1 gene therapy and BM-MNC implantation improved endothelium-dependent flow increase of the internal iliac artery. Ang-1 has been shown to stimulate the endothelial PI3-kinase-Akt (protein kinase B) pathway through the receptor tyrosine kinase Tie-2,20,21 which can enhance endothelial formation of nitric oxide (NO). We previously showed that endothelium-derived NO is necessary for endothelial migration and therefore ischemia-induced angiogenesis.46,47 Taken together, Ang-1 gene therapy might have not only facilitated angiogenesis but also maintained endothelial function (eg, NO release), which could further adapt to the rapid change of oxygen demand (ischemia) in vivo. Because Ang-1 mRNA expression is downregulated by hypoxia,48 a combination of Ang-1 with BM-MNCs may be an ideal combination to create organized vessels during tissue ischemia.

The present findings have several clinical implications. Therapeutic angiogenesis using plasmids encoding angiogenic genes or autologous BM-MNCs implantation have been already developed independently. Thus, combination therapy using Ang-1 plasmid gene and implantation of autologous BM-MNCs may be one of the most feasible strategies for therapeutic angiogenesis in the clinical arena in near future. Additionally, in the present study, far fewer BM-MNCs were used, ≈20% of the amount previously used for therapeutic angiogenesis in our laboratory. Nevertheless this low-dose regimen of BM-MNCs induced sufficient angiogenesis when combined with Ang-1 gene therapy. This would indicate that the combination of BM-MNCs and Ang-1 gene therapy would enable us to reduce the amount of the BM aspiration required to attain a sufficient degree of therapeutic angiogenesis.

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Disclosures
None.

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Materials and Methods

Rabbit BM-MNCs Isolation

All animal protocols were approved by the Institutional Animal Care and Use Committee of Nagoya University School of Medicine. Under anesthesia with ketamine (50 mg/kg) and xylazine (5 mg/kg), rabbit BM (5 mL) was aspirated from the right iliac crest. BM-MNCs were isolated by centrifugation through a Histopaque density gradient (Sigma, St. Louis, MO) as described previously.8,28

Rabbit Model of Unilateral Hind Limb Ischemia

Neovascular formation in response to tissue ischemia was examined using a rabbit model of unilateral hind limb ischemia.8,29 Male New Zealand White rabbits (2.5 to 3.0 kg) (Japan SLC, Hamamatsu, Japan) were anesthetized with ketamine (50 mg/kg) and xylazine (2.5 mg/kg). Through skin incision, all branches of the femoral artery were ligated with 4.0 silk (Braided Silk; AZWELL, Japan), then the femoral artery was excised from its proximal origin to the distal point where it bifurcates into the saphenous and popliteal arteries. This procedure resulted in retrograde propagation of thrombus and complete occlusion of the external iliac artery. Consequently, blood flow to the ischemic limb was entirely dependent on new collateral vessels issuing from the internal iliac artery.

Therapeutic Neovascularization with Angiopoietin-1 Plasmid and/or Autologous BM-MNCs
Rabbits \((n = 40)\) were subjected to unilateral hind limb ischemia and randomly divided into 4 groups. On day 7 after the induction of limb ischemia, systolic calf blood pressure measured by a sphygmomanometer (BP-98E, Softron, Tokyo) in ischemic limb was <40 mmHg in all rabbits, and there was no difference among the experimental groups. Rabbits received empty plasmid vector (pCA1) plus PBS injection (control group, \(n = 11\)), Ang-1 plasmid (pCAhAng-1) plus PBS injection (Ang-1 group, \(n = 11\)), pCA1 plus BM-MNC transplantation (BM-MNC group, \(n = 10\)), pCAhAng-1 plus BM-MNC transplantation (combination group, \(n = 8\)). In vivo transfer of Ang-1 plasmid gene and implantation of BM-MNCs were performed with a 26-gauge needle at 5 sites in 3 major thigh muscles of the ischemic limb. In brief, 100 \(\mu g\) of empty plasmid (pCA1) or Ang-1 plasmid (pCAhAng-1) per 0.5 mL of PBS was injected at 5 sites (total, 500 \(\mu g/2.5\) mL) on day 7 after the induction of limb ischemia. On day 10 (3 days after the injection of plasmid), autologous BM-MNCs \((1 \times 10^6)\) were suspended in 2.5 mL of PBS and transplanted at 5 sites in thigh muscles. Collateral vessel formation, microvascular angiogenesis and the functionality of blood flow in the ischemic hind limb were analyzed as described below.

**Gene Expression in the Rabbit Muscles after Hind Limb Ischemia**

Using RT-PCR analysis, we examined the expression of plasmid-specific Ang-1 mRNA in the rabbit muscles. On postoperative day 7, 500 \(\mu g\) of pCAhAng-1 was injected into thigh muscles. Rabbits were euthanized 3 days after plasmid injection and thigh muscles with plasmid injection were removed. Total RNA was
extracted and subjected to RT-PCR. Human-specific Ang-1 mRNA was detected using a specific primer set overlapping the human Ang-1 gene and the rabbit \(\beta\)-globin terminator of the pCAcc vector. The primers for human specific Ang-1 were for sense, 5'-CAGAGGCAGTACATGCTAAGAATTGAGTTA-3' (human Ang-1 specific primer), and for antisense, 5'-AGATGCTCAAGGGGCTTCATGATG-3' (rabbit \(\beta\)-globin terminator specific reverse primer). The size of the PCR product for human Ang-1/ rabbit \(\beta\)-globin terminator was 453 bp.

Furthermore, the efficiency of transgene expression was evaluated morphometrically with the use of a promoter-matched reporter plasmid, pCA\(\beta\), that encodes \(\beta\)-galactosidase. On postoperative day 7, 500 \(\mu\)g of pCA\(\beta\) was injected into thigh muscles. The transfected muscles were harvested 5 days later, fixed in 1% paraformaldehyde, and incubated with X-Gal (5-bromo-4-chloro-3-indolyl-\(\beta\)-D-galactopyranoside) chromogen (Invitrogen, Carlsbad, Calif) for 1 hours at 37°C.

**Iliac Angiography**

Formation of collateral vessels was evaluated by iliac angiography on postoperative day 35 as described previously. Angiograms were taken at exactly 3 sec after the internal iliac artery was filled with a nonionic contrast medium (Iopamiron 370; SCHERING, Berlin, Germany). We calculated the angiographic score as described previously. Angiographic luminal diameter of the internal iliac artery (stem collateral artery) in the ischemic hind limb was
measured at the site of the recording of the Doppler sample volume. The time period that the contrast medium flowed from the internal iliac artery to either the saphenous or popliteal arteries through the collateral vessels (contrast filling time) was also measured.

**Doppler Guide Wire Measurement**

A 0.014 inch Doppler flow wire (FloWire; Cardiometrics, Mountain View, CA) was advanced through the 4Fr catheter positioned at the lower abdominal aorta to the internal iliac artery supplying the ischemic limb. After recording resting average peak velocity (APV, the temporal average of the instantaneous peak velocity waveform), acetylcholine (1.5 µg/kg/min) was administered via the 4Fr catheter. After the evaluation of flow responses to acetylcholine, an intra-arterial bolus of nitroglycerin (50 µg/kg) was administered to assess endothelium-independent vasomotor reactivity. \(^{31,32}\) To evaluate endothelial function of newly formed vessels in the ischemic limb, the ratio of the endothelium-dependent flow increase (acetylcholine) to the endothelium-independent flow increase (nitroglycerin) was calculated.

**Transcutaneous Oxygen Pressure Measurements**

Transcutaneous oxygen pressure (TcO\(_2\)) was measured at an electrode temperature of 44 °C (TCM-400; Radiometer, Copenhagen, Denmark) and recorded after stabilization over a period of 10 min. Measurements were performed on the dorsum, crus and thigh of both hind limbs on day 35 and the
average TcO$_2$ value of the three skin points was calculated. The ischemic/normal hind limb TcO$_2$ ratio was calculated and was considered as a physiological parameter of the extent of functional blood perfusion.

**Capillary/Muscle Fiber Ratio**

Tissue specimens were obtained from the adductor and semimembranous skeletal muscles on day 35. Each of these two muscles was originally perfused by the deep femoral artery that was ligated when the common/superficial femoral arteries were excised. Frozen tissue sections with 5 $\mu$m thickness were prepared so that the muscle fibers were oriented transversely. The sections were stained for alkaline phosphatase to detect capillary endothelium. Eosin (0.5%) was used for counterstaining. Alkaline phosphatase-positive capillary endothelial cells were counted under light microscopy (x 200). Five fields from each sample were randomly selected for the counts. To ensure that the capillary density was not overestimated as a consequence of myocyte atrophy or underestimated because of interstitial edema, the capillary/muscle fiber ratio was also determined.

**Evaluation of Ischemic Skin Ulcer of Hind Limb**

On postoperative day 35, we evaluated the extent of skin necrosis/ulcer and limb loss in the ischemic hind limbs, and classified the magnitude as follows grade 0) no skin ulcer; grade 1) ulcer < 2 cm in longer diameter; grade 2) intermediary between grades 2 and 3; grade 3) ulcer expanding a half of crus in the major
axis; grade 4) auto-amputation of distal lower limb with bony exposure.

**In Vitro Assay for EPC function**

**EPC Migration Assay**

BM-MNCs were isolated from additional 6 rabbits and were cultured in Medium-199 supplemented with 20% fetal bovine serum (FBS), bovine pituitary extract (as endothelial growth supplements), heparin, and antibiotics (Invitrogen, Carlsbad, California; basal medium). BM-MNCs cultured on 6-cm plastic plates at a density of 2,000 cells/mm$^2$ were placed in a standard humidified incubator under 95% air and 5% CO$_2$. At day 7 of culture, adherent spindle-shaped cells were used in EPC migration assay as described previously.\textsuperscript{8,28} EPC-like adherent cells were stained with an anti-Tie-2 antibody (Santa Cruz Biotechnology, Santa Cruz, Calif) and secondary antibody with Alexa Fluor 488 (Molecular Probes). EPC migration in response to rhAng-1 and/or rhVEGF-A (R&D Systems, Minneapolis, MN) was analyzed using a modified Boyden chamber apparatus (Neuroprobe, Gaithersburg, MD) as described previously.\textsuperscript{28} Data were expressed as the number of migrated cells/microscopic field.

**Endothelial Network Formation in Basement Matrix Gel**

BM-MNCs were cultured at a density of 2,000 cells/mm$^2$ on 6-cm plastic plates in Medium-199 supplemented with 20% FBS and different growth factor conditions (control medium, rhAng-1 50 ng/mL, or rhVEGF 50 ng/mL). Matrigel tube formation assay was performed to assess the ability of BM-MNC derived EPCs...
to be incorporated into endothelial network structures, which is considered to be an important functional analysis for the process of vasculogenesis. On day 10 of BM-MNCs culture, spindle-shaped adherent cells ($6 \times 10^3$) were isolated from each plate and labeled with a green fluorescent dye PKH2-GL (Sigma) and co-cultured with unlabeled HUVECs ($6 \times 10^4$) on growth factor reduced Matrigel (Becton-Dickinson). Matrigel was placed in 12-well culture plates and incubated at $37^\circ$ for 12 hours with EGM-2 containing 5% FBS. Number of labeled EPCs incorporated into the HUVEC tube formation was assessed in 3 random high-power fields (x 100) per group.

**Ex Vivo Whole Mounts of the Mouse Ear Vasculature**

Male C57BL/6J mice (6 to 8 weeks old) were anesthetized, and rhVEGF (150 ng), rhAng-1 (150 ng) and littermate BM cells ($1.5 \times 10^5$) were injected into the posterior auricular muscle, midway up the dorsal aspect of the external ear, using a syringe with a 29-gauge needle. Recombinant protein suspended in 15 µL PBS was injected daily for 3 days, and BM cells of littermate in 15 µL PBS were implanted on the first day. Mice were euthanized with an overdose of pentobarbital 4 days after the first injection. The entire vascular network of the ear was visualized after intravascular vital staining with a fluorescein-labeled *Lycopersicon esculentum* lectin (Vector Laboratories, Burlingame, CA) that binds the luminal surface of all blood vessels as previously described. In brief, mice were anesthetized and fluorescein-labeled lectin was injected from the tail vein, and 2 minutes later the tissues were fixed by perfusion of 1%
paraformaldehyde in PBS. Ears were then removed, bisected in the plane of the cartilage. Whole mounts of the ear skin were analyzed using a confocal laser scanning microscope (MRC-1024; BIO-RAD, San Diego, CA) and photographed.

**Statistics**

Results are expressed as mean ± SEM. Statistical significance of differences was analyzed among experimental groups by ANOVA followed by Bonferroni’s test for comparison between any 2 groups. Multiple comparisons in nonparametric analysis were performed by the Kruskal-Wallis test. Correlation coefficients were calculated using Spearman rank correlation. Statistical significance was assumed at a $P$ value less than 0.05. $n$ represents the number of animals.
**Supplementary figure _plugin**. (A) Structure of human Ang-1 expression plasmid (pCAhAng-1). CMV, cytomegalovirus; ori, the origin of replication; Amp<sup>R</sup>, β-lactamase gene. (B) pCAhAng-1-specific mRNA expression in thigh muscles of ischemic hind limb. There is no transgene mRNA expression in control vector-injected muscles (lane 2), but there is a clear expression of pCAhAng-1-specific mRNA in skeletal muscles with Ang-1 gene therapy (lane 3) GAPDH expression as an internal control is shown in the lower panel. (C) Expression of promoter-matched reporter plasmid, pCAβ, encoding for β-galactosidase. Positive expression is identified as dark-blue staining of muscle fiber. Macroscopic and microscopic findings reveal that transfection efficiency is higher when plasmid was injected in ischemic muscle. Yellow bar = 5 mm. Black bar = 100 μm.

**Supplementary figure _plugin**. Physiological parameters of collateral blood flow were improved by the combination of Ang-1 gene and BM cell implantation therapy. (A) Endothelial function of the internal iliac artery as assessed by the ratio of the Ach-/NTG-induced flow increase tended to be increased in the combination group compared to the other three groups. (B) Filling time of contrast medium between the internal iliac artery and the bifurcation point of the femoral artery was significantly shorter in the combination group than in the control and Ang-1 groups.
**A**

![Diagram of a plasmid with the following components: CMV enhancer, β-actin promoter, β-actin 1st exon, β-globin terminator, pUC ori, AmpR, h Angiopoietin-1, pCA1 / hAng1 5694 bp, β-actin/β-globin intron, PCR product.]

**B**

Transgene Expression

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**C**

Supplementary figure
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