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**Original**

# Mesenchymal Stem Cells from Bone Marrow Enhance Neovascularization and Stromal Cell Proliferation in Rat Ischemic Limb in the Early Phase after Implantation

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**Abstract**: Accumulating evidence from animal studies shows that the administration of mesenchymal stem cells (MSCs) from adult bone marrow ameliorates tissue damage after ischemic injury. In the present study we investigated the efficacy of MSC implantation into a hindlimb ischemia model over a short-term period to elucidate the effects conferred within the early phase after treatment. MSCs from rats expressing green fluorescence protein (GFP) were injected into rat ischemic limbs. Laser Doppler perfusion imaging revealed significantly higher blood perfusion recovery in the MSC group than in the control group on days 3 and 7 after the treatment. The capillary / muscle fiber ratio in ischemic muscle was also significantly higher in the MSC group than in the controls in a histological study. In spite of these benefits, we found no evident engraftment of the GFP-positive cells, and instead, the MSC treatment induced a proliferation of resident stromal cells in the perivascular area of the ischemic muscle, some of which produced vascular endothelial growth factor. The present study suggested that MSC therapy promotes neovascularization even in the early phase, both directly through endothelial proliferation and indirectly through activation of the resident stromal cells.

**Key words**: mesenchymal stem cells, limb ischemia, angiogenesis

## Introduction

Chronic critical limb ischemia (CLI) is the most severe manifestation of peripheral artery disease[1,2]. CLI is a sometimes fatal condition that profoundly diminishes quality of life and global function. Therapeutic angiogenesis with bone marrow mononuclear cells was developed as a less invasive intervention for patients severely compromised by CLI[3], aimed

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at improving perfusion to the ischemic vascular beds by inducing the formation of new blood vessels from preexisting vessels. However, recent studies indicated that this bone marrow-based transplantation therapy fails to confer sufficient effects on limb salvage in atherosclerotic peripheral artery disease[4,5], and a new cell therapy using other cell types that will hopefully prove more efficacious is expected in the near future.

Mesenchymal stem cells (MSCs) from adult bone marrow possess attractive properties for use in safe and effective cardiovascular cell therapies[6]. MSCs can be easily isolated from bone marrow and expanded in culture systems, and they express a number of pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF), as well as proteins that modulate endothelial cell migration[7,8]. Our group previously examined an MSC culture system to confirm the angiogenic and endothelial protective effects of expressed cell factors[7,9]. A co-culture with human MSCs induced prominent capillary network formation in our *in-vitro* angiogenesis assay[9].

Previous studies have demonstrated the mid- to long-term benefits of MSC implantation on limb perfusion in a rodent limb ischemia model[10,11], but the effects conferred within the early phase after treatment remain to be fully understood. In this study we investigated the efficacy of MSC therapy in the short time frame just after implantation.

## Methods

*Isolation and culture of MSCs from GFP-rat bone marrow*

We isolated and cultured MSCs from the bone marrow of adult transgenic Sprague-Dawley rats ubiquitously expressing enhanced green fluorescence protein (GFP), as previously described[12]. In brief, mononuclear cells were purified from GFP-rat bone marrow by density centrifugation and resuspended in a complete culture medium (CCM) consisting of alpha-MEM (Life Technologies, Carlsbad, CA) with 20% fetal calf serum (Atlantic Biologicals, Norcross, GA), 100 U / ml penicillin, 100 μg / ml streptomycin, and 1 mM L-glutamine. The cells were plated in 20 ml of medium in a 150-$cm^2$ culture dish and incubated in a humidified incubator with 95% air and 5% $CO_2$ at 37℃. After 24 h, the non-adherent cells were removed and the primary adherent cells were cultured and propagated.

For cell implantation, the MSCs (passages 5 to 7) were plated in CCM with a change of medium every 4 days until the cultures reached 70～80% confluence. The cells thus prepared were harvested and injected into rat ischemic limbs.

*Rat hindlimb ischemia model and cell implantation*

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. The Animal Care and Use Committee of Showa University approved the experimental protocol.

Hindlimb ischemia was induced in 8-week-old male Wister rats by ligating the right femoral arteries under anesthesia. The distal portion of the saphenous artery and all of the side branches were ligated, along with the veins. The left hindlimb was kept intact and used as a non-ischemic limb. After the operation, the rats were injected with either MSCs ($5\times10^5$ cells,

n=11) or vehicle (control, n=11) into the ischemic adductor muscle at four sites.

Blood perfusion was assessed by laser Doppler perfusion imaging (LDPI) (Omega Zone, Muromachi, Tokyo, Japan) on days 3 and 7 after the operation. The blood flow distribution of the limb was mapped out as a color-coded image of intensity directly proportional to the blood flow perfusion. The LDPI index was used to calculate the blood perfusion ratio of the ischemic and non-ischemic hindlimbs. Tissue samples were obtained from rat ischemic adductor muscles at 7 days after surgery for immunohistochemistry.

*Quantification of capillary vessels and proliferating cells*

Immunohistochemistry for CD31 and Ki67 was performed to detect capillary vessels and proliferating cells, respectively, in rat ischemic limbs as previously described[9]. The sections (5 μm) were deparaffinized and subjected to a 3-step staining procedure using streptavidin-biotin complex with horseradish peroxidase. Horseradish peroxidase activity was visualized with diaminobenzidine substrate, and the sections were faintly counterstained with hematoxylin. The primary antibodies used were raised against CD31 (1:100 dilution, DAKO, Carpinteria, CA) and Ki67 (1:100 dilution, Abcam, Cambridge, UK) proteins.

Three fields from each tissue section (n=6) were randomly selected and CD31-positive cells were counted in each field. To avoid over- or underestimating the capillaries as a consequence of myocyte atrophy or interstitial edema, the capillary number adjusted per muscle fiber was used to compare differences in capillary density. The proliferating cell index was defined as the ratio of Ki67-positive cells to the total number of cells in each perivascular interstitial area.

*Immunofluorescence*

Immunofluorescence was performed as previously described[7]. Tissue sections were incubated with anti-GFP (1:400, Life Technologies) and anti-VEGF (1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA), and then with secondary antibodies conjugated to Alexa 488 (1:1000). Slides were mounted in Vectashield containing 4', 6-diamidino-2-phenylindole (Vector Labs).

*Data analysis*

All data were expressed as mean ±SD. Student's t-test was used to compare differences between groups. Probability values of $<0.05$ were considered significant.

## Results

*Enhanced neovascularization in rat ischemic limbs by MSC implantation*

Bone marrow MSCs from GFP rats ($5\times10^5$ cells) were injected into rat ischemic limbs, and blood perfusion recovery of the ischemic hindlimb was evaluated by LDPI. Hindlimb ischemia was markedly improved in the MSC group (Fig. 1), with the quantitative analysis of LDPI findings revealing significantly higher perfusion recovery compared to rats in the control group on days 3 and 7 after treatment.

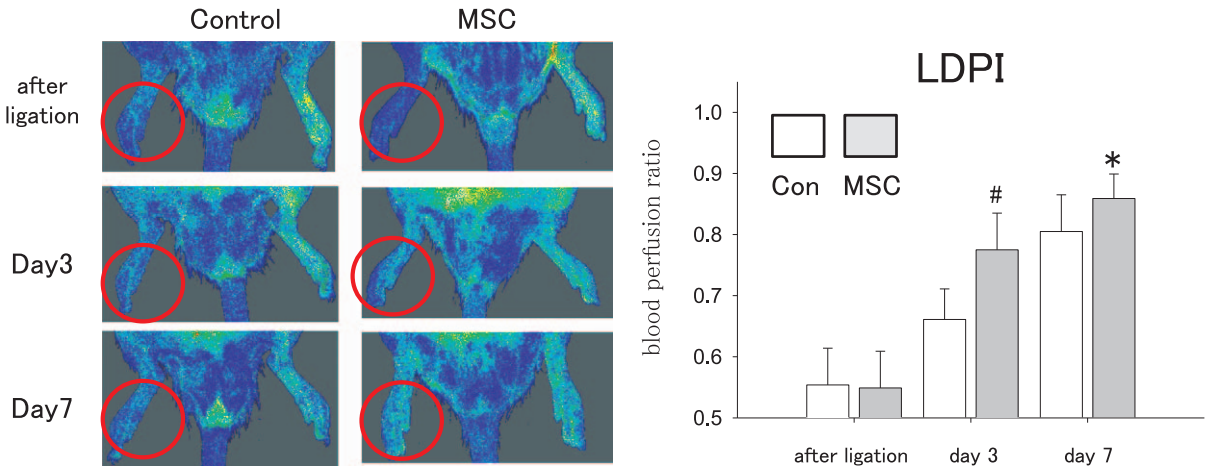


Fig. 1. Blood flow recovery in rat ischemic muscle. Upper, Representative images of laser Doppler blood flow in the control and MSC groups. Blood perfusion signals of the ischemic limb were higher in the MSC-treated limb compared than in the controls on days 3 and 7. Lower, The ischemic / normal laser Doppler blood flow ratio was significantly higher in the MSC group than in the control group (Con) in the quantitative analysis. n=11 in each group; #, $P < 0.001$ on day 3; *, $P < 0.05$ on day 7. LDPI, laser Doppler perfusion imaging.

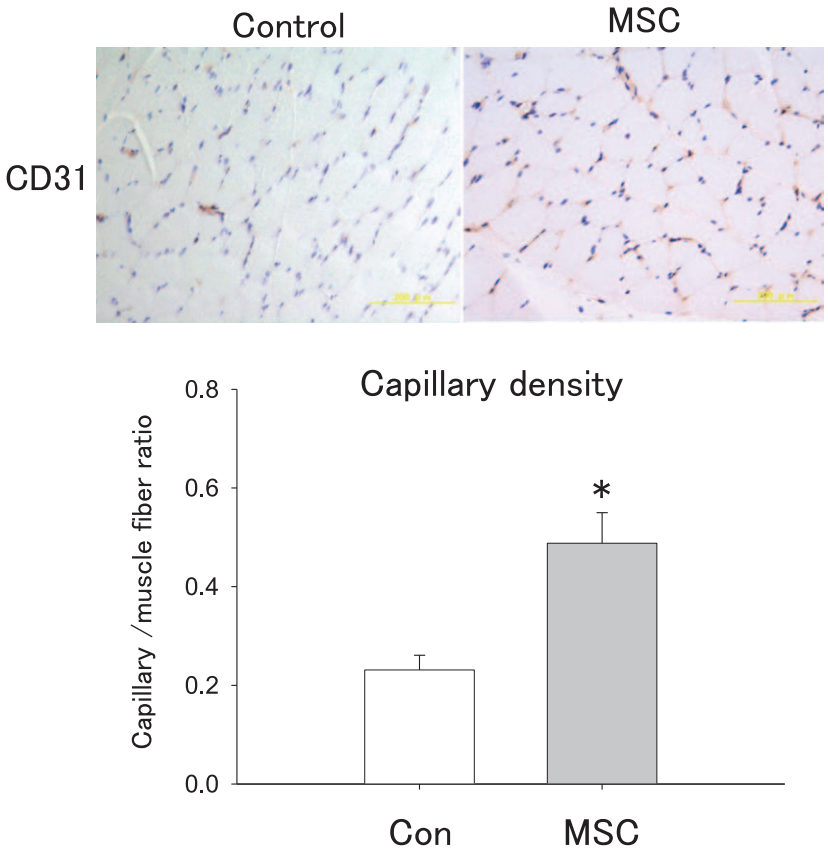


Fig. 2. Capillary density in rat ischemic muscle. Upper, Immunohistochemical staining for CD31 in muscle sections on day 7 after the treatment. Lower, The capillary / muscle fiber ratio was significantly higher in the MSC group than in the control group (Con) by quantitative analysis. Bar=200 μm. n=6 in each group; *, $P<0.05$.

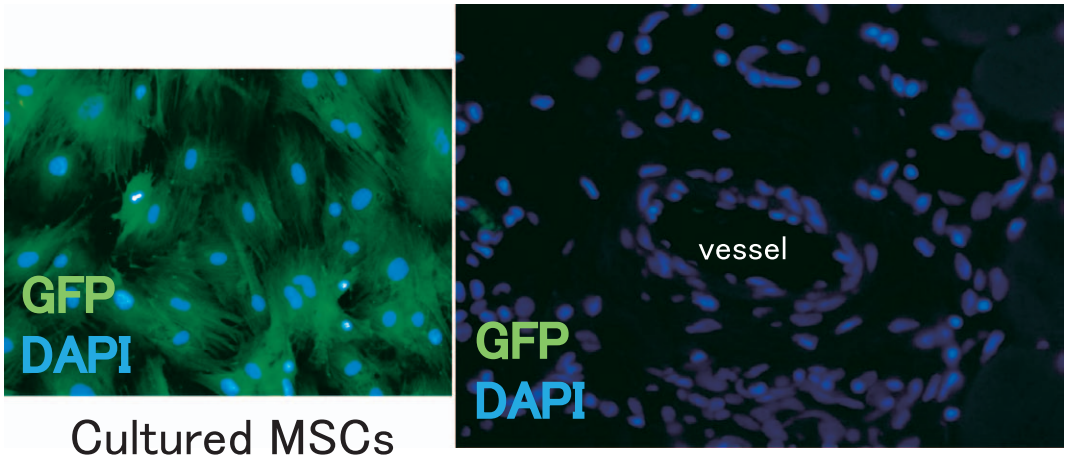


Fig. 3. No engraftment of MSCs from GFP rats in ischemic muscle. Left, Microscopic image of GFP-positive MSCs in the culture system. Right, Representative sections showing immunofluorescence for GFP (green). Blue fluorescence indicates DAPI nuclear staining. No GFP-positive cells were engrafted into the ischemic muscle. GFP, green fluorescence protein ; DAPI, 4',6-diamidino-2-phenylindole.

In the histological study, more capillary vessels were detected in the ischemic muscle treated with MSCs than in the control group on day 7 after the implantation (Fig. 2). The capillary / muscle fiber ratio in ischemic muscle was also significantly higher in the MSC group than in controls.

Thus, local MSC administration into the ischemic limb significantly enhanced neovascularization even in the early phase after implantation.

*MSC engraftment after the implantation*

Immunohistochemical assessment of the extent of MSC engraftment revealed no MSCs (GFP signal) in the ischemic muscles of MSC-treated rats at 1 week after implantation (Fig. 3). In an earlier study, we also found that MSCs exerted favorable cardiac effects after myocardial infarction without long-term engraftment or differentiation[7]. The benefits demonstrated in that study were attributable to transitory paracrine effects or secreted factors following the MSC administration. In the present study we similarly found that MSCs promoted neovascularization in ischemic limb muscle without persistent engraftment.

*Stromal cell proliferation in muscle*

In addition to enhanced angiogenesis, we also observed an increased number of Ki67-positive cells in the perivascular interstitial area of the ischemic muscle treated implanted with MSCs (Fig. 4), and quantitative analysis indicated a significantly higher ratio of proliferating stromal cells in the MSC group than in controls. Interestingly, while the MSC implantation induced stromal cell proliferation, fibrosis of the hindlimb muscle was less extensive after ischemic injury and seemed not to differ between the groups on day 7 (data not shown).

The absence of GFP-positive cells indicated that the proliferating stromal cells were derived

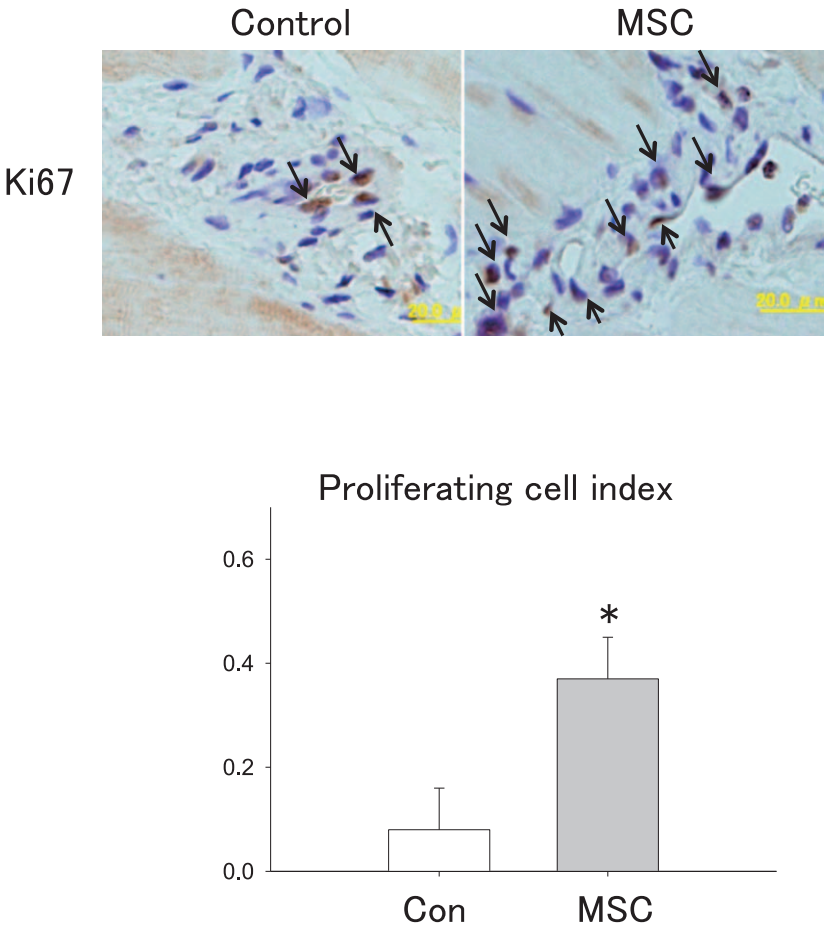


Fig. 4. Proliferating stromal cells in ischemic muscle. Upper, Immunohistochemical staining for Ki67, a cell proliferation marker, in muscle sections on day 7 after the treatment. Ki67-positive cells were detected in the perivascular interstitial area. Lower, By quantitative analysis, the ratio between the number of Ki67-positive cells and the total number of cells in the perivascular interstitial area was significantly higher in the MSC group than in the control group (Con). Arrows indicate Ki67-positive cells. Bar=20 μm. n=6 in each group; *, $P < 0.05$.

from a source other than the injected MSCs. As it turned out, the stromal cells in the perivascular interstitial area expressed VEGF (Fig. 5).

Together, the findings suggested that the enhanced neovascularization by MSC implantation was at least partly attributable to the activation of resident stromal cells with angiogenic potential.

## Discussion

MSCs from bone marrow significantly promoted neovascularization in rat ischemic limbs in the early phase after implantation without cell engraftment, and the effect could be attributed to paracrine action. We previously reported improved cardiac function in the absence of long-

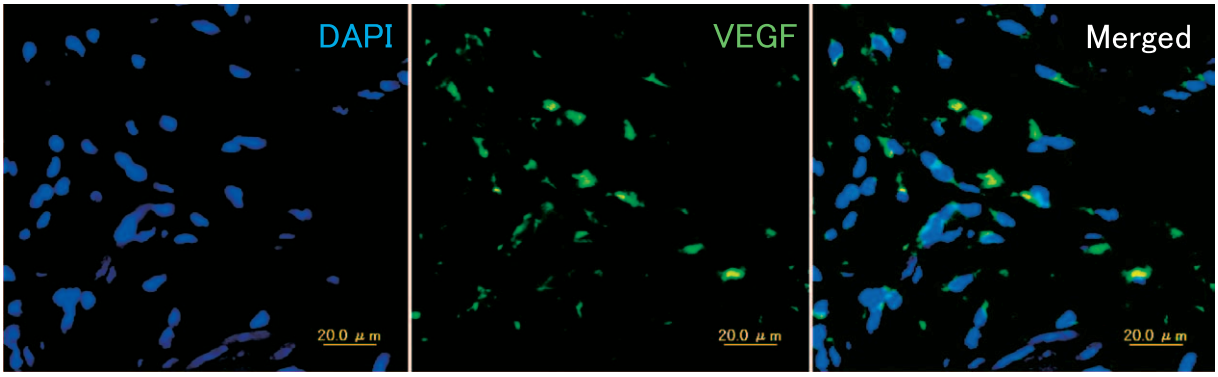


Fig. 5. Vascular endothelial growth factor (VEGF) expression of perivascular stromal cells in rat ischemic muscle. Green fluorescence indicates VEGF. Blue fluorescence indicates DAPI nuclear staining. Microscopic observation revealed that some of the perivascular stromal cells produced VEGF. Bar= 20 μm, DAPI, 4',6-diamidino-2-phenylindole.

term engraftment with systemic administration of human MSCs into immunodeficient mice with myocardial infarction[7]. In that study, the MSC-conditioned medium from the same donor protected both cultured murine cardiomyocytes and human umbilical vein endothelial cells from cell death during hypoxia. Consistent with the present results, the MSC-conferred benefits for cardiac repair also appeared to derive from a transitory paracrine action or from factors secreted by the cells.

Factors secreted from MSCs alter the growth, differentiation, survival, and / or function of endogenous resident cells even though they derive from a disparate tissue source[13, 14]. Several studies from other groups support the paracrine hypothesis for MSC-mediated angiogenesis and cardioprotection[10, 11, 15, 16]. MSCs are a robust source of angiogenic factors[7, 8, 10], and in experiments comparing cultured human MSCs with hematopoietic stem cells, the former expressed higher mRNA levels for pro-angiogenic factors such as VEGF and adrenomedullin[7], while conditioned medium from the MSCs rescued human umbilical vein endothelial cells from cell death during hypoxia exposure. We have also observed an *in-vivo* vasculoprotective effect of conditioned medium from MSCs in a mouse model of myocardial infarction[17]. Implanted MSCs apparently protect endothelial cells and induce their proliferation in ischemic tissue via secretion of several angiogenic factors.

Another important finding in the present study was the enhanced proliferation of stromal cells in the perivascular area of the MSC treated-ischemic limbs. MSCs secrete basic fibroblast growth factor (bFGF) as well as VEGF[7, 9], and bFGF released from MSCs promotes wound healing via stromal cell migration and proliferation[18, 19]. MSCs also stimulate both the proliferation and neurogenesis of endogenous neural progenitor cells in the hippocampus after intracranial administration[20]. The administration of peripheral blood mononuclear cells also induced the activation and marked proliferation of myoblasts in ischemic skeletal muscle, resulting in coordinated muscle regeneration and enhanced neovascularization[21]. We therefore assumed that stem / progenitor cells from one tissue could be used to influence the in situ

activation of not only endothelial cells, but also the resident primitive cells from a different adult tissue.

Some of the stromal cells in ischemic muscle produced VEGF in the present study. In tumor biology, tumor angiogenesis is augmented by the induction of VEGF in perivascular stromal cells[22], thus the stromal cells in skeletal muscle are likely to be one of the resident cells with angiogenic potential. We can thus hypothesize that the MSCs not only promote the endothelial proliferation directly, but also induce neovascularization indirectly via the activation of perivascular stromal cells in a paracrine fashion. Our previous study likely supports this hypothesis, in that MSC therapy significantly increased VEGF levels in remote areas wholly devoid of MSCs in a porcine myocardial infarction model[9]. In these experiments, the MSC-treated heart contained clusters of resident interstitial cells positive for phospho-STAT3, a regulator of VEGF production. However, stromal cell populations are heterogeneous, and VEGF expression was apparently absent in some of the single stromal cells. Further studies will be necessary to explore the stromal cell subpopulation responsible for angiogenesis in endogenous reparative processes.

In conclusion, a salutary effect of the MSC implantation could be demonstrated within 3 days after treatment, and the efficacy was sustained without persistent cell engraftment. The present study suggested that MSC therapy promotes neovascularization even in the early phase, both directly via endothelial proliferation and indirectly via activation of the resident stromal cells in a paracrine fashion by factors secreted from the MSCs.

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