

Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia

Takashi Iwase^{a,b}, Noritoshi Nagaya^{a,*}, Takafumi Fujii^c, Takefumi Itoh^a, Shinsuke Murakami^a,
Toshio Matsumoto^b, Kenji Kangawa^d, Soichiro Kitamura^e

^aDepartment of Regenerative Medicine and Tissue Engineering, National Cardiovascular Center Research Institute,
5-7-1 Fujishirodai, Suita, Osaka 565-8565, Japan

^bDepartment of Medicine and Bioregulatory Sciences, University of Tokushima Graduate School of Medicine, Tokushima, Japan

^cDepartment of Cardiac Physiology, National Cardiovascular Center Research Institute, Osaka, Japan

^dDepartment of Biochemistry, National Cardiovascular Center Research Institute, Osaka, Japan

^eDepartment of Cardiovascular Surgery, National Cardiovascular Center, Osaka, Japan

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Abstract

Objective: Mesenchymal stem cells (MSC) are pluripotent cells that differentiate into a variety of cells including endothelial cells and vascular smooth muscle cells. Although transplantation of bone marrow-derived mononuclear cells (MNC) has already been applied for the treatment of critical limb ischemia, little information is available regarding comparison of the angiogenic potency between MSC and MNC. Accordingly, we injected equal numbers of MSC or MNC in a rat model of hindlimb ischemia and compared their therapeutic potential.

Methods and results: Immediately after creating hindlimb ischemia, rats were randomized to receive MSC transplantation (MSC group), MNC transplantation (MNC group), or vehicle infusion (Control group). Three weeks after transplantation, the laser Doppler perfusion index was significantly higher in the MNC group than in the Control group (0.69 ± 0.1 vs. 0.57 ± 0.06 , $P < 0.01$). Furthermore, there was a marked improvement in blood perfusion in the MSC group (0.81 ± 0.08). Capillary density was highest in the MSC group. The number of transplanted cell-derived endothelial cells was higher in the MSC group than in the MNC group. Transplanted cell-derived vascular smooth muscle cells were detected only in the MSC group. In vitro, MSC were more tolerant to apoptotic stimulus (serum starvation and hypoxia) than MNC.

Conclusions: MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well under an ischemic environment, and differentiated into not only endothelial cells but also vascular smooth muscle cells. Thus, MSC transplantation may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

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Keywords: Angiogenesis; Apoptosis; Cell therapy

1. Introduction

Peripheral vascular disease is a major health care problem in an aging society [1]. In the late stages of

peripheral vascular disease, progression of tissue hypoperfusion results in ischemic ulceration and gangrene. Unfortunately, amputation is required in more than a third of these patients. Transplantation of bone marrow-derived mononuclear cells (MNC) has been shown to induce therapeutic neovascularization in critical limb ischemia [2–4]. Thus, MNC have been established as a tool for cell therapy. However, MNC transplantation requires harvesting a large

* Corresponding author. Tel.: +81 6 6833 5012; fax: +81 6 6833 9865.

E-mail address: nnagaya@ri.ncvc.go.jp (N. Nagaya).

amount of bone marrow under general anesthesia, which may impose a load on some patients with severe complications such as myocardial ischemia, heart failure, cerebral disease, or renal failure.

Mesenchymal stem cells (MSC), which reside in the bone marrow stroma, possess pluripotency and differentiate into osteoblasts, chondrocytes, neurons, skeletal muscle cells, endothelial cells and vascular smooth muscle cells [5–7]. MSC have an adherent nature and are expandable in culture. Thus, it would be easy to obtain a sufficient number of MSC for cell therapy. MSC have been shown to form capillary-like structures in an in vitro Matrigel assay [8]. Furthermore, MSC transplantation has been shown to induce neovascularization in a rat model of hindlimb ischemia [9]. These findings suggest that, like MNC, transplantation of MSC may have beneficial effects in patients with critical limb ischemia. However, there has been no study on the therapeutic potency of MSC compared with that of MNC.

Thus, the purposes of this study were (1) to compare the therapeutic potencies of MSC and MNC transplantation in a rat model of hindlimb ischemia and (2) to investigate the mechanisms underlying the angiogenic potential of MSC.

2. Methods

2.1. Animal model of hindlimb ischemia

Male Lewis rats weighing 250 to 275 g were used in this study. These isogenic rats served as donors and recipients to simulate autologous implantation of MSC or MNC. To create a hindlimb ischemia model, the left common iliac artery of each rat was resected under anesthesia with pentobarbital sodium (50 mg/kg i.p.). The distal portion of the saphenous artery and all side branches as well as veins were dissected free and excised. The right hindlimb was kept intact and used as a nonischemic limb. All protocols were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.2. Expansion of MSC and isolation of MNC

MSC expansion was performed as described previously [7]. In brief, we sacrificed male Lewis rats and harvested bone marrow by flushing the cavity of the femurs and tibias with phosphate-buffered saline (PBS). Bone marrow cells were introduced into 100-mm dishes and cultured in α -MEM supplemented with 10% FBS and antibiotics. A small number of cells developed visible symmetric colonies by days 5 to 7. Nonadherent hematopoietic cells were removed and the medium was replaced. The adherent, spindle-shaped MSC population expanded to over 50 million cells at approximately 4 to 5

passages after first culturing the cells. MNC were isolated from whole marrow cells by Ficoll density gradient centrifugation (Lymphoprep, Nycomed) as described previously [3].

2.3. MSC and MNC transplantation

Immediately after resection of the left common iliac artery, 30 rats were randomized to the following three groups: (1) MSC transplantation (MSC group, $n=10$), (2) MNC transplantation (MNC group, $n=10$), and (3) PBS injection (Control group, $n=10$). In each group, 5×10^6 MSC, 5×10^6 MNC or PBS was injected into the ischemic thigh muscle with a 26-gauge needle at five different points.

2.4. Assessment of blood perfusion

A laser Doppler perfusion image (LDPI) analyzer (Moor Instruments) was used to measure serial blood flow over a period of 3 weeks. Low or no blood perfusion was displayed as dark blue, whereas the highest perfusion was displayed as red. After blood flow had been scanned twice, the average flow values of the ischemic and nonischemic limbs were calculated by computer-assisted quantification using stored images. The LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion [10].

Blood flow of the ischemic hindlimb was also assessed by use of colored microspheres 3 weeks after transplantation as reported previously [11,12]. In brief, 2×10^6 microspheres (Dye-Trak, Triton Technology) were injected into the left ventricle under anesthesia and artificial ventilation. Reference blood samples were withdrawn from the right carotid artery at a rate of 0.3 ml/min. Rats were then killed, and muscle samples from the adductor, semimembranous and gastrocnemius muscles were harvested and weighed. These samples were processed according to the manufacturer's instructions, and the absorbance of dye from muscle samples (AU_{muscle}) and reference blood samples (AU_{sample}) was measured with a spectrophotometer. The regional blood flow of ischemic muscle was calculated as $0.3 \times (AU_{\text{muscle}}) / (AU_{\text{sample}})$ and presented normalized per 100 g body weight.

2.5. Histological assessment

Rats were killed 3 weeks after transplantation. Four pieces of ischemic tissue from the adductor and semimembranous muscles were obtained and snap-frozen in liquid nitrogen. Frozen tissue sections were stained with alkaline phosphatase using an indoxyltetrazolium method to detect capillary endothelial cells [3,10]. Five fields from four tissue sections were randomly selected, and the number of capillaries was counted in each field. To avoid overestimation or underestimation of capillary density as a result of myocyte atrophy or interstitial edema, the

capillary number adjusted per muscle fiber was used to compare the differences in capillary density among the three groups [3].

2.6. Monitoring of transplanted MSC or MNC in ischemic hindlimb muscle

An additional 10 rats were used to examine whether transplanted MSC or MNC differentiate into endothelial cells or vascular smooth muscle cells in ischemic muscle. Suspended MSC and MNC were labeled with fluorescent dye (PKH26 Red Fluorescent Cell Linker Kit, Sigma Chemical) as reported previously [13]. Red fluorescence-labeled MSC (5×10^6 cells per animal) or MNC (5×10^6 cells per animal) were transplanted into the ischemic thigh muscle in rats (MSC and MNC groups, $n=5$ each). This subgroup of rats was killed 3 weeks after MSC or MNC transplantation, and frozen tissue sections were obtained from the ischemic muscle. The tissue sections were incubated with anti-von Willebrand factor polyclonal antibody (vWF, DAKO) or anti- α -smooth muscle actin monoclonal antibody (α SMA, DAKO), followed by

incubation with Alexa Fluor® 633 IgG antibody (Molecular Probes) or fluorescein isothiocyanate (FITC)-conjugated IgG antibody (BD Pharmingen), respectively. Five high power fields ($40\times$) of each section were randomly selected to count the number of transplanted cells, vWF-positive cells, and α SMA-positive cells.

Green fluorescent protein (GFP)-expressing MSC or MNC were also transplanted to examine cell differentiation. MSC and MNC were isolated from male GFP-transgenic rats, which were provided by Dr. Masaru Okabe (Osaka University, Japan) [14,15]. GFP-expressing MSC (5×10^6 cells per animal) or MNC (5×10^6 cells per animal) were transplanted into the ischemic thigh muscle in male Spargue–Dawley rats (MSC group, $n=3$; MNC group, $n=3$). Immunohistochemical analysis for vWF was performed 3 weeks after cell transplantation.

2.7. Cell viability assay

MSC or MNC were plated in serum-free medium on 12-well plates (1×10^5 cells per well) and cultured in hypoxic conditions (1% O_2 /5% CO_2 /94% N_2) for 24 h.

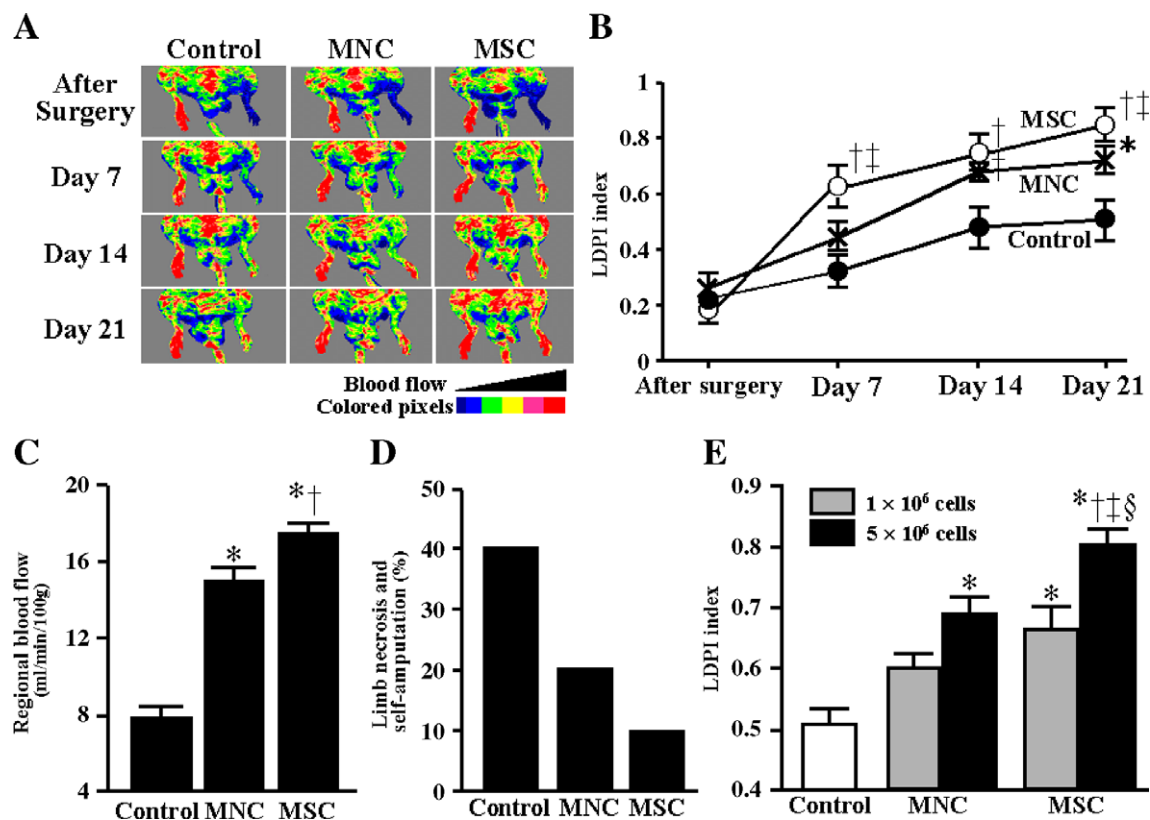


Fig. 1. (A) Representative examples of serial LDPI. Blood perfusion of the ischemic hindlimb markedly increased in the MSC group 3 weeks after transplantation (red to orange). (B) Quantitative analysis of hindlimb blood perfusion. LDPI index was determined as the ratio of ischemic to nonischemic hindlimb blood perfusion. The increase in the LDPI index was not significant in the Control group. LDPI index was significantly higher in the MNC and MSC groups than in the Control group 3 weeks after surgery. LDPI index in the MSC group was highest among the three groups. Data are mean \pm S.E.M. * $P < 0.05$ and $^{\dagger}P < 0.01$ vs. Control; $^{\ddagger}P < 0.01$ vs. MNC. (C) Blood flow assessment of ischemic muscle by use of microspheres. Data are mean \pm S.E.M. * $P < 0.01$ vs. Control; $^{\dagger}P < 0.05$ vs. MNC. (D) Incidence of limb necrosis and self-amputation 3 weeks after transplantation. (E) Comparison between the number of transplanted cells and perfusion recovery of the ischemic hindlimb. LDPI index of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. Data are mean \pm S.E.M. * $P < 0.01$ vs. Control; $^{\dagger}P < 0.01$ vs. 1×10^6 MNC; $^{\ddagger}P < 0.05$ vs. 5×10^6 MNC; $^{\S}P < 0.05$ vs. 1×10^6 MSC.

Hypoxic conditions were obtained by the use of a CO₂/multigas incubator (ASTEC). After exposure to serum-free hypoxia, the cells were suspended using trypsin and mixed with 0.4% trypan blue solution (Sigma Chemical). Percentages of viable cells were evaluated by two blinded observers and normalized to the total cell number in the field.

2.8. *In vitro* apoptosis assay

Terminal dUTP nick-end labeling (TUNEL) assay (ApopTag Fluorescein kit, Serological Corporation) was performed to evaluate apoptosis of MSC or MNC induced by the combination of serum starvation and hypoxia. After incubation for 24 h, MSC or MNC were fixed in 1% paraformaldehyde, and TUNEL was performed for detection of apoptotic nuclei according to the manufacturer's instructions. The cells were then mounted in medium containing 4',6-diamidino-2-phenylindole (DAPI). Randomly selected microscopic fields ($n=5$) were evaluated to calculate the ratio of TUNEL-positive cells to total cells.

2.9. Western blot analysis of hypoxia-inducible factor (HIF)

To identify the protein expression of HIF-1 α , Western blotting was performed using a mouse monoclonal antibody raised against HIF-1 α (clone H1 α 67, Novus Biologicals). Serum-starved MSC or MNC were cultured in normoxic or hypoxic conditions for 24 h. Cells were

homogenized on ice in 150 μ l lysis buffer with a protease inhibitor. Then 15 μ g of protein was transferred to sample buffer, loaded on 7.5% SDS-polyacrylamide gel, and blotted onto a polyvinylidene fluoride membrane. After being blocked for 60 min, the membrane was incubated with a primary antibody in blocking buffer (1:1000), followed by incubation with a peroxidase-conjugated secondary antibody. Positive protein bands were visualized by chemiluminescence using an ECL kit (Amersham). Western blot analysis using a monoclonal antibody raised against β -actin (Sigma Chemical) was used as a protein loading control.

2.10. *In vitro* tube formation assay

In vitro Matrigel assay was performed to investigate whether MSC or MNC induce tube formation under the condition of serum-free hypoxia [6,8]. MSC or MNC were plated in serum-free medium on 12-well plates (1×10^6 cells per well) coated with Matrigel (Becton Dickinson). After 6-h incubation in hypoxia, tube formation was examined with a phase-contrast microscope.

2.11. Measurements of angiogenic factors

To compare the secretion of angiogenic factors from MSC with that from MNC, a total of 1×10^6 MSC or MNC were plated in serum-free medium on 6-well plates. After 24-h incubation, the conditioned medium was

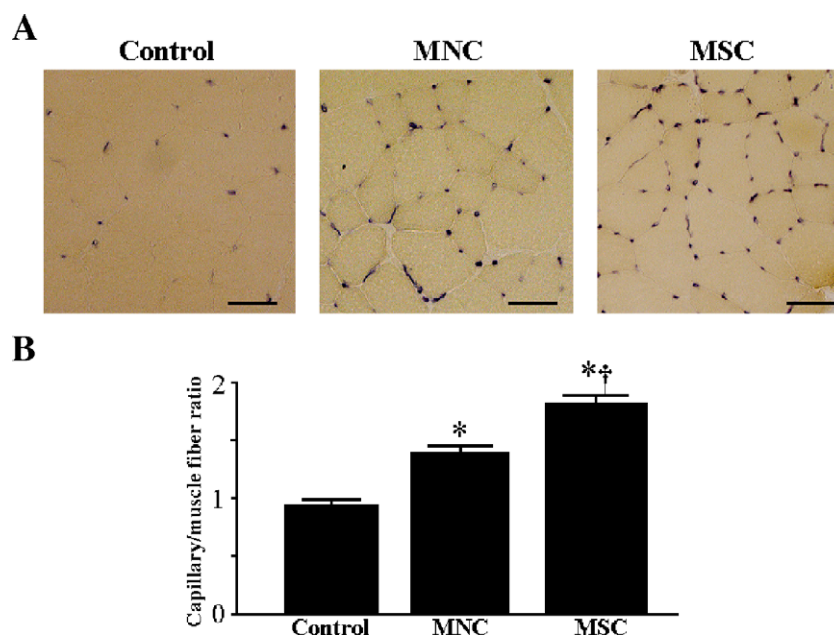


Fig. 2. (A) Representative examples of alkaline phosphatase staining in ischemic hindlimb muscles. In the MNC and MSC groups, the number of capillaries markedly increased compared with the Control group. (B) Quantitative analysis of capillary density in ischemic hindlimb muscles. Capillary density is shown as capillary/muscle fiber ratio. The capillary/muscle fiber ratio of ischemic hindlimb muscle was highest in the MSC group, followed by the MNC group and Control group. Data are mean \pm S.E.M. * $P < 0.01$ vs. Control; † $P < 0.01$ vs. MNC. Scale Bars: 50 μ m.

collected and, levels of VEGF, basic FGF (bFGF), and stromal cell-derived factor-1 α (SDF-1 α) were measured using enzyme immunoassay kits (Quantikine, R&D Systems).

2.12. Statistical analysis

All values are expressed as mean \pm S.E.M. Comparisons of parameters among the three groups were made by one-way ANOVA followed by Scheffe's multiple comparison test. Student's unpaired *t*-test was used to compare differences between two groups. A probability value <0.05 was considered statistically significant.

3. Results

3.1. Blood perfusion of ischemic hindlimb after transplantation

Blood perfusion of the ischemic hindlimb was considerably impaired 3 weeks after surgery (Control group, Fig. 1A). On the other hand, improvement of hindlimb ischemia was observed in the MNC and MSC groups compared with the Control group. Hindlimb ischemia was markedly improved in the MSC group. Quantitative analysis demonstrated that the LDPI index was highest in the MSC group, followed by the MNC group and

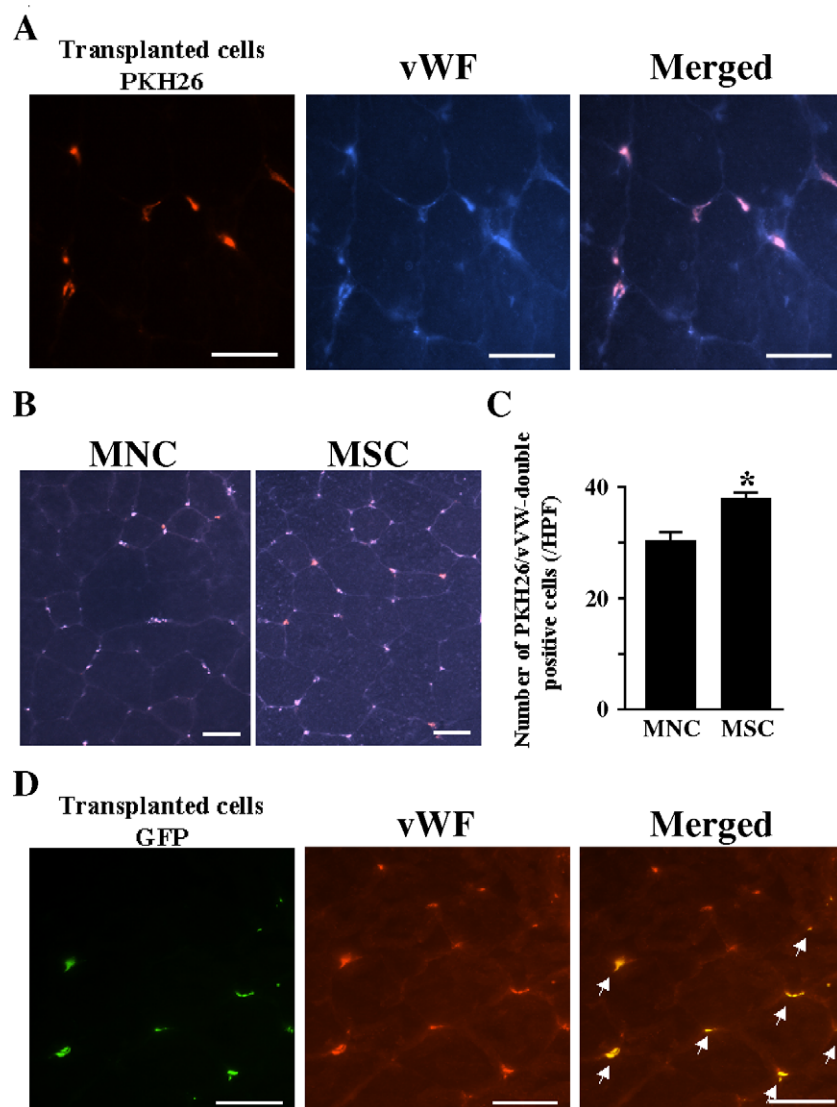


Fig. 3. Endothelial differentiation of transplanted cells. (A) Incorporation and differentiation of transplanted MSC in ischemic thigh muscle. Red fluorescence (PKH26)-labeled MSC were incorporated into interstitial tissues between muscle fibers and were positive for vWF (blue). (B) PKH26/vWF-double-positive cells (pink) were frequently observed in the MSC group. (C) The number of PKH26/vWF-double-positive cells (MNC-derived endothelial cells) was significantly higher in the MSC group than in the MNC group. Data are mean \pm S.E.M. **P*<0.01 vs. MNC. Bars: 50 μ m. (D) Representative photographs of MSC-derived vascular structures. Green fluorescent protein (GFP)-expressing MSC were transplanted into ischemic thigh muscle. GFP (green)/vWF (red)-double positive cells (orange, arrows) were observed 3 weeks after transplantation. Bars: 50 μ m.

Control group (Fig. 1B). The regional blood flow of ischemic muscle was highest in the MSC group, followed by the MNC group and Control group (Fig. 1C). The incidence of limb necrosis and self-amputation of ischemic hindlimb was minimal in the MSC group, followed by the MNC group and Control group (Fig. 1D).

To examine the relationship between the number of transplanted cells and their angiogenic potential, a smaller number of MSC or MNC (1×10^6 cells per animal) was transplanted into the ischemic thigh muscle ($n=10$ each). Transplantation of 1×10^6 MNC tended to improve blood perfusion of the ischemic hindlimb 3 weeks after transplantation, but these changes did not reach statistical significance. On the other hand, transplantation of 1×10^6 MSC significantly improved hindlimb ischemia. The LDPI index of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation (Fig. 1E).

3.2. Capillary density of ischemic hindlimb

A large number of capillaries were detected in the ischemic muscle of the MSC and MNC groups (Fig. 2A). Importantly, quantitative analysis demonstrated that the capillary/muscle fiber ratio of ischemic muscle was highest in the MSC group, followed by the MNC group and Control group (Fig. 2B).

3.3. Endothelial differentiation of transplanted cells

Red fluorescence-labeled MSC and MNC were detected in the interstitial tissues between muscle fibers 3 weeks after transplantation (Fig. 3A). PKH26-positive cells expressed

vWF, an endothelial marker, in both the MSC and MNC groups. Importantly, PKH26/vWF-double-positive cells were frequently observed in the MSC group (Fig. 3B). Quantitative analysis demonstrated that the number of PKH26/vWF-double-positive cells was significantly higher in the MSC group than in the MNC group (Fig. 3C). GFP-expressing MSC and MNC were also detected in the ischemic muscle and were positive for vWF 3 weeks after transplantation (Fig. 3D). The number of GFP/vWF-double-positive cells was significantly higher in the MSC group than in the MNC group (data not shown).

3.4. Differentiation of transplanted cells into vascular smooth muscle cells

Some of the transplanted MSC were positive for α SMA, a marker of vascular smooth muscle cells. They participated in the formation of vascular structures as mural cells. In contrast, none of the MNC was stained by α SMA (Fig. 4).

3.5. Cell survival under serum starvation and hypoxia

After 24-h incubation under serum-free and hypoxic conditions, the majority of cultured MNC revealed cytoplasmic shrinkage, disintegration into small vesicles, and membrane blebbing (Fig. 5A). In contrast, these morphological changes were rarely observed in MSC. The percentage of dead cells, assessed by the trypan blue exclusion test, was significantly higher in MNC than in MSC (Fig. 5B). TUNEL staining showed that serum-free hypoxia markedly induced MNC apoptosis, whereas only a small number of MSC were TUNEL-positive (Fig. 5C). The

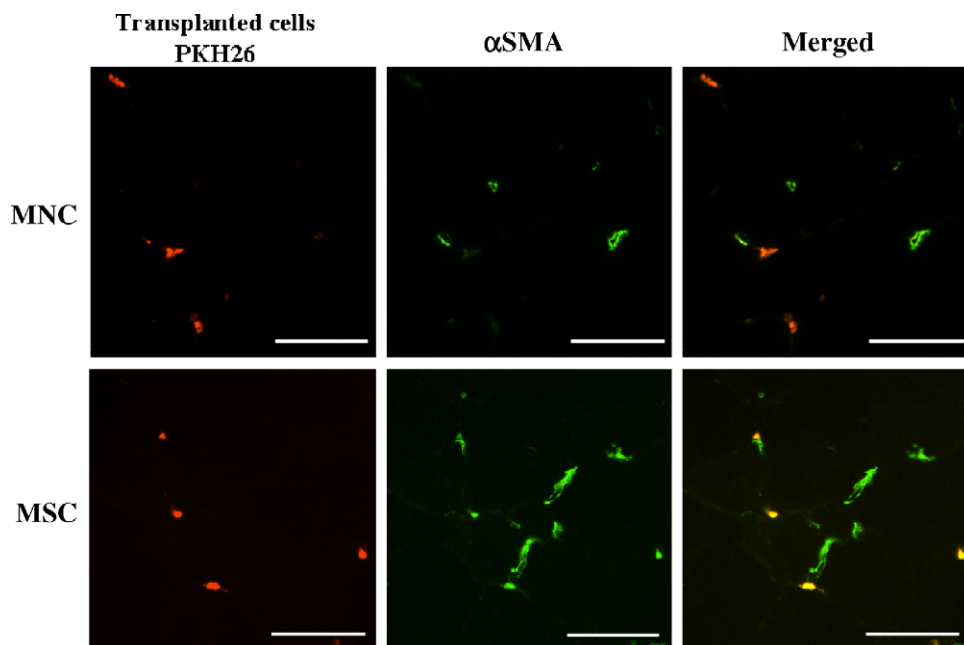


Fig. 4. Differentiation of transplanted cells into vascular smooth muscle cells. Red fluorescence (PKH26)-labeled MSC were stained by α SMA, whereas transplanted MNC did not express α SMA. Bars: 50 μ m.

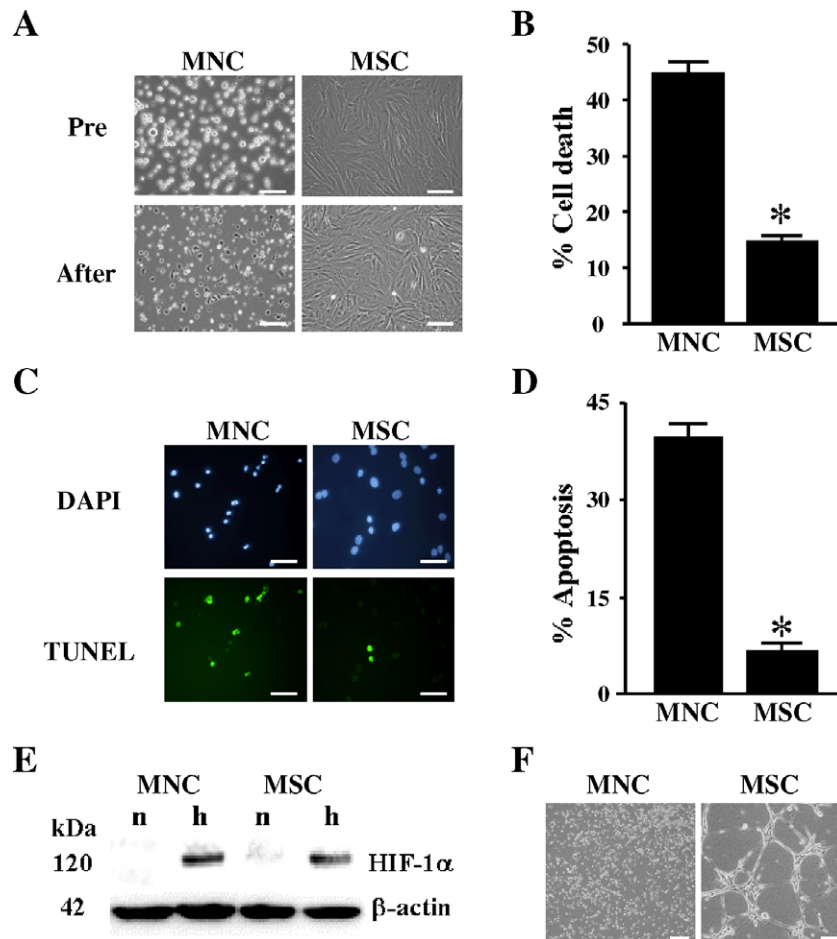


Fig. 5. (A–D) In vitro cell viability assay and apoptosis assay. (A) Representative photographs of MSC and MNC. (B) Quantitative analysis of cell viability by trypan blue staining. The percentage of dead cells in MSC was significantly lower than that in MNC. (C) Representative photographs of apoptotic MSC and MNC. Apoptosis of MSC or MNC was detected by TUNEL staining (green). Nuclei were stained with DAPI (blue). Serum starvation and hypoxia substantially induced MNC apoptosis. (D) Quantitative analysis of TUNEL-positive cells. The percentage of TUNEL-positive cells in MNC was significantly higher than that in MSC. Data are mean \pm S.E.M. * $P < 0.01$ vs. Control. (E) Western blot analysis for hypoxia-inducible factor (HIF)-1 α and β -actin. The expression of HIF-1 α protein was not detected in MSC and MNC under the condition of normoxia (n). However, HIF-1 α protein was expressed in both cell types after exposure to serum-free hypoxia (h). (F) Representative photographs of in vitro Matrigel assay. After 6-h incubation in serum-free hypoxia, MSC formed typical tube-like structures. In contrast, MNC did not show any morphological change. Bars: 100 μ m.

percentage of TUNEL-positive cells was significantly higher in MNC than in MSC (Fig. 5D). The expression of HIF-1 α protein was observed in both MSC and MNC under serum-free and hypoxic conditions (Fig. 5E). The ratios of HIF-1 α / β -actin did not significantly differ between MSC and MNC (data not shown).

3.6. Tube formation under serum starvation and hypoxia

After 6-h incubation on Matrigel, tube formation was observed in MSC, whereas MNC did not show any morphological change (Fig. 5F).

3.7. Secretion of angiogenic factors from MSC and MNC

VEGF and bFGF were detected in conditioned medium of cultured MSC and MNC. Compared with MNC, MSC secreted significantly greater amounts of VEGF and bFGF

(VEGF: 817 ± 36 vs. 188 ± 32 pg/ 10^6 cells, $P < 0.01$; bFGF: 47 ± 5 vs. 4 ± 1 pg/ 10^6 cells, $P < 0.01$). Although SDF-1 α was not detected in conditioned medium of MNC, MSC secreted a large amount of SDF-1 α (17 ± 1 ng/ 10^6 cells).

4. Discussion

In the present study, we demonstrated that (1) transplantation of MSC as well as MNC induced angiogenesis in a rat model of hindlimb ischemia, (2) the extent of neovascularization was significantly greater in MSC transplantation than in MNC transplantation, (3) transplanted MSC highly differentiated into endothelial cells compared with transplanted MNC, and (4) only MSC differentiated into vascular smooth muscle cells in ischemic tissue. We also demonstrated in vitro that (5) MSC were more tolerant to an ischemic stimulus than

MNC and that (6) MSC secreted large amounts of angiogenic factors compared with the amounts secreted by MNC.

Earlier studies have shown that MNC transplantation enhances neovascularization by supplying endothelial progenitor cells and multiple angiogenic factors such as VEGF, bFGF, and angiopoietin-1 [3,4,16]. In fact, MNC transplantation significantly augmented blood perfusion and capillary density in the ischemic hindlimb in the present study. Other studies have shown that transplanted MSC differentiate into endothelial cells, secrete angiogenic factors, and thereby induce neovascularization in ischemic tissue [9,17,18]. However, it remains unclear whether the angiogenic potency of MSC transplantation is comparable or superior to that of MNC transplantation. In the present study, we injected equal numbers of MSC or MNC into ischemic muscle to compare the therapeutic effects of the two types of cells. Interestingly, MSC transplantation markedly increased blood perfusion and capillary density in the ischemic hindlimb compared with MNC transplantation. Moreover, perfusion recovery of 1×10^6 MSC transplantation was equivalent to that of 5×10^6 MNC transplantation. These results suggest that MSC transplantation is more potent in therapeutic angiogenesis than MNC transplantation.

The underlying mechanisms responsible for the superiority of MSC in therapeutic angiogenesis remain unknown. Earlier studies have shown that many transplanted cells undergo apoptosis immediately after transplantation because of a lack of oxygen and nutrition, although they should survive for a sufficiently long period to induce angiogenesis [3,19]. In fact, the present study showed in vitro that MNC readily underwent cell death and apoptosis under conditions of serum starvation and hypoxia. These findings raise the possibility that the therapeutic potency of transplanted MNC is considerably attenuated by an ischemic environment. In contrast, MSC survived well under these conditions. Thus, MSC may be more appropriate for cell transplantation with respect to cell survival than MNC.

The present study showed that transplanted MSC and MNC participated in vascular structures and expressed vWF, an endothelial cell marker. The number of MSC-derived vWF-positive cells in ischemic muscle was significantly higher than that of MNC-derived vWF-positive cells. Previous studies have shown that both transplanted MSC and MNC are capable of differentiating into endothelial cells in ischemic tissue [3,9]. However, the present study showed that a combination of serum starvation and hypoxia greatly reduced MNC viability. Furthermore, only MSC induced tube formation in serum-free and hypoxic conditions. Taking these results together, it is interesting to speculate that transplanted MSC survive well and differentiate into endothelial cells in an ischemic environment and thereby induce angiogenesis more efficiently than transplanted MNC.

During the process of neovascularization, vascular smooth muscle cells play an important role in vessel maturation [20,21]. In the present study, none of the transplanted MNC expressed α SMA, which is consistent with recent findings that MNC-derived CD34-positive cells rarely expressed a vascular smooth muscle cell marker and highly differentiated into endothelial cells in ischemic muscle [22]. On the other hand, earlier studies have shown that MSC readily acquire vascular smooth muscle properties in vitro and that transplanted MSC differentiate into vascular smooth muscle cells in ischemic tissue [9,23]. The present study also demonstrated that some transplanted MSC were positive for α SMA, a vascular smooth muscle cell marker, and formed vascular structures as mural cells. Thus, unlike MNC, transplanted MSC may contribute to vessel maturation.

Recent studies have demonstrated that the angiogenic potential of MSC and MNC is attributed not only to their differentiation into vascular endothelial cells but also to their ability to produce various angiogenic factors, including VEGF and bFGF [4,16–18]. The present study demonstrated that MSC secreted large amounts of VEGF and bFGF compared with the amounts secreted by MNC. Interestingly, only MSC significantly secreted SDF-1 α , which also has been shown to induce angiogenesis in vivo and in vitro [24,25]. These findings suggest that MSC transplantation induces angiogenesis more efficiently than MNC transplantation partly through the release of angiogenic factors.

From a clinical standpoint, MNC transplantation is considered to be an established procedure that is easy to implement without any immunosuppressive agents and expensive facilities [2,4,26–31]. In contrast, MSC transplantation requires time and considerable cost to obtain an adequate number of MSC under strictly aseptic conditions. Nevertheless, MSC are an attractive source for cell therapy because they are easily isolated from a small amount of bone marrow and rapidly expand in culture. Thus, MSC transplantation may be one of the most attractive cell therapies in the treatment of critical limb ischemia.

In conclusion, MSC transplantation caused significantly greater improvement in hindlimb ischemia than MNC transplantation. Compared with MNC, MSC survived well in an ischemic environment and differentiated into not only endothelial cells but also vascular smooth muscle cells. Thus, MSC transplantation may be a new therapeutic strategy for the treatment of severe peripheral vascular disease.

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