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Hypoxic Preconditioning Enhances Angiogenic Potential of Bone Marrow Cells With Aging-Related Functional Impairment

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Background: Hypoxic preconditioning of bone marrow cells (BMCs) from young healthy individuals can enhance the cells' therapeutic potential. Considering that the response to hypoxia may differ according to the quality of the cells, we assessed the effect of hypoxic preconditioning on BMCs from aged mice and compared the difference in response between BMCs from aged and young mice.

Methods and Results: BMCs from young (3 months) and aged (20–22 months) mice were subjected to hypoxic preconditioning by culture for 24 h in 2% O_2. Compared with BMCs from young mice, those from aged mice showed significantly fewer CD34- or c-kit-positive stem cells, higher expression of p53, and lower telomerase activity. Adhesion, survival and angiogenic potency were also lower in BMCs from aged mice, indicating an aging-related impairment. Hypoxia-preconditioned BMCs from aged mice showed enhanced adhesion, survival, and angiogenic potency with the in vitro assessments, as well as the in vivo implantation into ischemic hindlimbs. All the enhancements by hypoxic preconditioning were comparable between BMCs from aged and young mice, although the angiogenic potential of BMCs with and without hypoxic preconditioning was lower in old mice compared with young mice.

Conclusions: Similar responses to hypoxia by BMCs from both aged and young mice suggest that hypoxic preconditioning could be a useful method of enhancing the angiogenic potential of BMCs. (*Circ J* 2012; **76:** 986–994)

Key Words: Aging; Angiogenesis; Cell therapy; Hypoxia; Preconditioning

Therapeutic angiogenesis induced by the implantation of autologous bone marrow-derived cells is becoming a new approach to treating several ischemic diseases. Although clinical trials utilizing cell-based therapeutic angiogenesis have been safely conducted, the therapeutic benefits seem to be relatively modest and are not observed in all patients.[1–4] Many methods, including gene modification and ex vivo expansion of the cells before implantation, have been tested in an effort to augment therapeutic efficacy.[5–10] However, applying these methods to the clinical setting can be difficult to implement, expensive, and time-consuming. Therefore, simpler and faster methods of enhancing the efficacy of cells used for therapeutic angiogenesis need to be explored.

Editorial p 823

We have successfully developed a simple method of enhancing the therapeutic potential of cell-based angiogenesis through short-term exposure of the cells to low oxygen, termed "hypoxic preconditioning".[11–13] Studies from other groups[14–18] as well as our own have demonstrated that ex vivo hypoxic preconditioning of bone marrow-derived stem cells or cardiac stem cells before implantation can enhance the expression of various proangiogenic factors, cellular adhesion molecules, and antiapoptotic and antioxidative factors. These changes following hypoxic preconditioning improve cell survival and angiogenic potency after implantation into ischemic tissue. Unfortunately, these exciting data were achieved using cells

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from healthy young animals as donors and recipients,[11–18] but the patients who would undergo cell-based therapy generally present with advanced age and systemic diseases such as diabetes. The response to low oxygen can vary according to the type and characterization of the cells. Given that previous studies have shown that advanced age and systemic diseases can contribute to functional impairment of bone marrow-derived stem cells,[19–28] it follows that exposure of these functionally impaired cells to hypoxic conditions might induce cellular apoptosis or other damage.

In this study, using aged mice, we investigated the impact of hypoxic preconditioning on the in vitro function of bone marrow cells (BMCs) and their in vivo efficacy for inducing therapeutic angiogenesis. We also examined whether there was a difference in the response to hypoxic preconditioning between BMCs from aged and young mice.

Methods

Animals

For these experiments, we used young (3 months) and aged (20–22 months) male C57BL/6 mice (Japan SLC, Shizuoka, Japan), as described previously.[23,29] All animal procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University.

Isolation of BMCs

Bone marrow from young and aged mice was collected from the femur and tibia, and mononuclear BMCs were isolated by density gradient centrifugation, as described previously.[11,30] Isolated BMCs were used for subsequent analysis.

Flow Cytometry Analysis

The expression of CD34 and c-kit in freshly isolated BMCs from young and aged mice was measured by flow cytometry. Briefly, cells were stained with a fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD34 antibody (RAM34, eBioscience Inc, San Diego, CA, USA) or a phycoerythrin (PE)-conjugated rat anti-mouse c-kit antibody (2B8, eBioscience Inc). Respective isotypes (eBioscience Inc) were used as negative controls. Quantitative flow cytometry analysis was performed using a FACSCalibur with CellQuest Pro software (BD Biosciences, Franklin Lakes, NJ, USA).

Western Blot Analysis

To examine the expression of p53, a marker of cell senescence, in freshly isolated BMCs from young and aged mice, we performed western blot analysis. Cells were lysed in a buffer [20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% Triton X-100, and complete protease inhibitor mixture tablets (Roche, Mannheim, Germany)], and centrifuged at 1,000 g for 10 min at 4°C. Protein concentrations of the cell lysates were determined using a BCA protein assay reagent kit (PIERCE, Rockford, IL, USA). The cell lysates (30 μg/well) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The blotted membranes were incubated with primary antibodies to p53 (PAb240, Abcam, Cambridge, UK) or β-actin (Sigma, St Louis, MO, USA), as an internal control. Corresponding horseradish peroxidase-conjugated antibodies were used as the second antibody (DakoCytomation, Carpinteria, CA, USA). Signals were visualized with an enhanced chemiluminescence western blot detection system (GE Healthcare UK Ltd, Buckinghamshire, UK) and recorded with FluorChem FC2 (Alpha Inotech Corp, San Leandro, CA, USA). Each band was quantified using Image J software, and the level of p53 was normalized to that of β-actin. Data are expressed as the fold increase relative to freshly isolated BMCs from young mice.

Telomeric Repeat Amplification Protocol Assay

The telomerase activity in freshly isolated BMCs from young and aged mice was measured using TeloTAGGG Telomerase PCR ELISA^{PLUS} kits (Roche). Proteins (1 μg) of each whole-cell lysate were used for elongation/amplification, and telomerase activity was quantified by ELISA, according to the manufacturer's instructions. Data are expressed as the fold increase relative to freshly isolated BMCs from young mice.

Hypoxic Preconditioning of BMCs

Isolated BMCs were collected and suspended at a density of 5×10^6/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. BMCs were exposed to hypoxic preconditioning for 24 h at 33°C in 2% O_2, 5% CO_2, and 93% N_2 (hypoxia-preconditioned), as described previously.[11–13] Normoxia-cultured (24 h at 33°C in 95% air, 5% CO_2) BMCs were used as a control.

Cell Viability Analysis

Immediately after 24 h of culture under normoxic or hypoxic conditions, or 1 day after an additional treatment with 100 μmol/L H_2O_2 at 37°C in 95% air, 5% CO_2, cell viability was determined by trypan blue dye exclusion as described previously.[12,30] The cell survival rate was calculated as the percentage of surviving cells among all the seeded cells.

Apoptosis Assay

The apoptosis assay was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences), according to the manufacturer's instructions. Briefly, after 24 h of culture under normoxic or hypoxic conditions, BMCs were collected, washed, and resuspended in 100 μl of annexin V binding buffer. The cells were incubated with 5 μl of annexin V-FITC and 5 μl of propidium iodide for 15 min at room temperature in the dark. Next, the cell suspension was supplemented with 400 μl of annexin V binding buffer and analyzed by flow cytometry.

Adhesion Analysis

Adhesion analysis was performed as described previously.[13] After 24 h of culture under normoxic or hypoxic conditions, BMCs were collected, washed, and resuspended in RPMI 1640 containing 10% FBS and 1% penicillin-streptomycin. Cells (2×10^6/ml per well) were then re-seeded onto 24-well culture plates coated with 8 μg/ml fibronectin (Invitrogen, Carlsbad, CA, USA) and incubated at 37°C in 95% air, 5% CO_2. After 1 day of culture, non-adherent cells were removed by tapping and gently washing the wells three times with phosphate-buffered saline (PBS). The number of adherent cells was counted in 5 random microscopic fields (×200-fold magnification) per well. Data are expressed as the number of adherent cells/field.

Analysis of Angiogenic Potency

To address the effect of hypoxic preconditioning on the angiogenic potency of BMCs, cells cultured under normoxic or hypoxic conditions for 24 h were collected, washed, and re-seeded at a density of 2×10^6/ml onto 4-well chamber culture slides (Nalge Nunc International, Naperville, IL, USA) coated with fibronectin. After incubation at 37°C in 95% air, 5% CO_2, we evaluated the secretion of vascular endothelial (VE) growth factor (VEGF) and the endothelial differentiation in

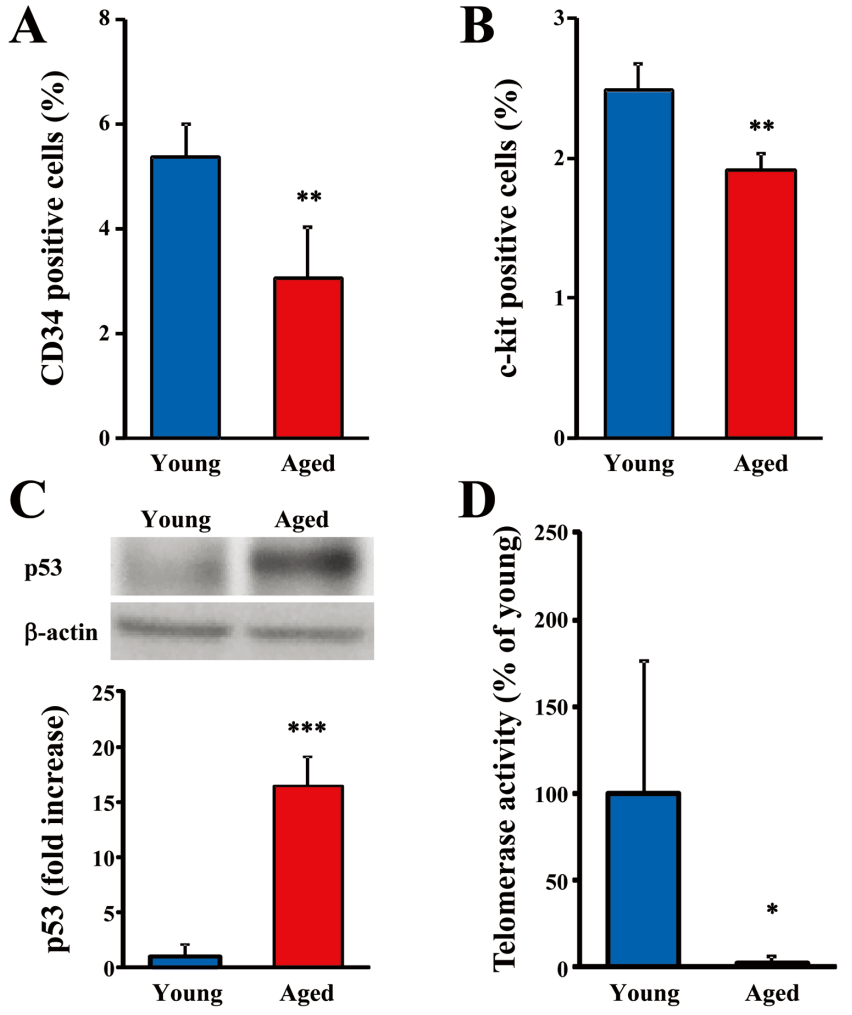

Figure 1. Characterization of bone marrow cells (BMCs). Freshly isolated BMCs from young and aged mice were used for flow cytometric analysis (**A**,**B**), western blot analysis (**C**), and telomeric repeat amplification protocol assay (**D**). The numbers of CD34 (**A**) and c-kit (**B**) positive stem cells in BMCs were lower in aged mice than in young mice. The expression of p53 was higher (**C**) and telomerase activity was lower (**D**) in BMCs from aged mice. *P<0.05, **P<0.01 and ***P<0.001 vs. cells from young mice (n=3 or 4).

the BMCs.

To estimate the VEGF production of the BMCs, we collected the supernatants 3 days after cultivation as described previously.[30] The concentration of VEGF in the supernatants was determined by VEGF ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Endothelial differentiation in BMCs was assessed by immunostaining for VE-cadherin as described previously.[27] Briefly, after 7 days of culture, the cells were fixed in acetone and blocked with Protein Block Serum-free (DakoCytomation, Inc). The cells were incubated with a PE-conjugated anti-mouse VE-cadherin antibody (1:50; C-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA). After the cells were washed, 4 random microscopic fields (×200-fold magnification) in each chamber were selected and the numbers of positively stained cells were counted. Experiments were repeated in 3 independent cultures. Data are expressed as the number of positively stained cells/field.

Ischemic Hindlimb Model and Cell Implantation

The mouse model of hindlimb ischemia and the cell implantation were performed as described previously.[12,13,30] Briefly, after the aged mice were given general anesthesia, the left femoral artery was exposed and ligated, and its branches were dissected free and excised. The quadriceps and adductor muscles of the ischemic hindlimbs were injected intramuscularly at 4 sites (10 μl PBS or 1×10^6 cells/point) with 1 of the following: PBS injection only (n=6); 4×10^6 freshly isolated BMCs from young mice (n=5); 4×10^6 normoxia-cultured BMCs from young mice (n=6); 4×10^6 hypoxia-preconditioned BMCs from young mice (n=6); 4×10^6 freshly isolated BMCs from aged mice (n=6); 4×10^6 normoxia-cultured BMCs from aged mice (n=6); or 4×10^6 hypoxia-preconditioned BMCs from aged mice (n=6).

Histological Analysis of Microvessel Density

Mice were euthanased 14 days after treatment, at which time the quadriceps and adductor muscles were harvested. To detect the development of microvessels in the ischemic muscles, frozen sections were stained for alkaline phosphatase with an indoxyl tetrazolium method, as described previously.[11,12,30] A total of 15 different fields (×200-fold magnification) on 3 independent slides from different cross-sections were randomly selected for each mouse, and the number of microvessels was counted. The density of microvessels was estimated by the microvessel/muscle fiber ratio.

Blood Flow in the Ischemic Limbs

Blood flow in the ischemic hindlimb was measured using a laser Doppler perfusion imaging system (PeriScan System, Perimed AB, Stockholm, Sweden) 14 days after treatment, as

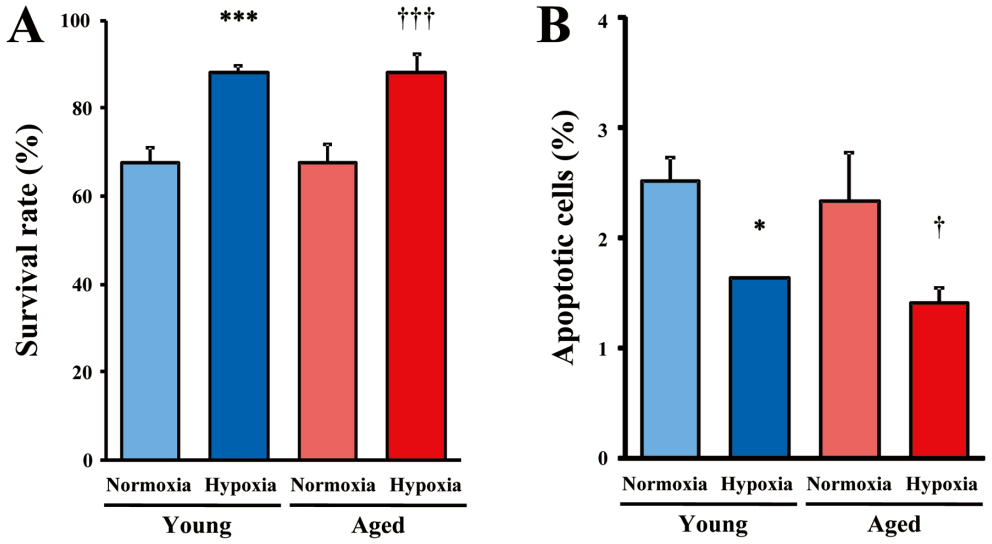

Figure 2. Cell viability and apoptosis of bone marrow cells (BMCs) after hypoxic preconditioning. BMCs from young and aged mice were cultured under normoxic or hypoxic conditions for 24h, and cell viability (**A**) and apoptosis (**B**) were determined. *P<0.05 and ***P<0.001 vs. normoxia-cultured cells from young mice; †P<0.05 and †††P<0.001 vs. normoxia-cultured cells from aged mice (n=3).

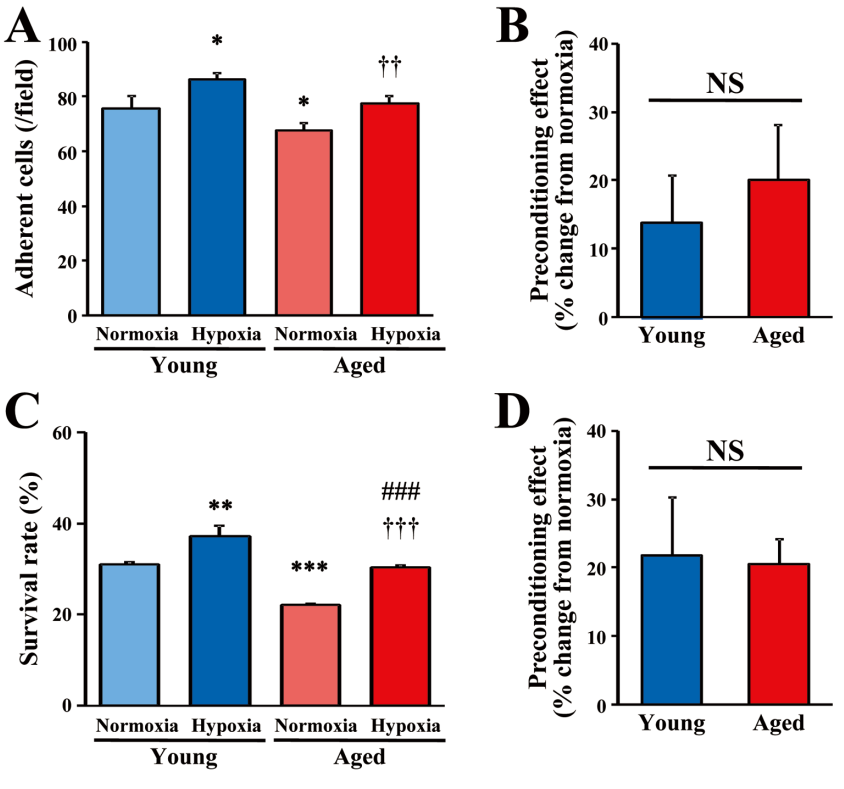

Figure 3. Effects of hypoxic preconditioning on adhesion and resistance to oxidative stress in bone marrow cells (BMCs). Hypoxia-preconditioned and normoxia-cultured cells from young and aged mice were cultured for 1 day, and adhesion (**A**,**B**) and survival (**C**,**D**) were assessed. (**A**,**B**) Adhesion of BMCs after culture. Adhesion capacity of the hypoxia-preconditioned cells was higher than that of the normoxia-cultured cells (**A**), and the effect of preconditioning on enhancing adhesion was similar between young and aged mice (**B**). (**C**,**D**) Survival rate of BMCs after treatment with 100 μmol/L H_2O_2. Under oxidative stress, the survival rate was higher for hypoxia-preconditioned cells than for normoxia-cultured cells (**C**), and the preconditioning effect in aged mice was comparable to that of young mice (**D**). *P<0.05, **P<0.01 and ***P<0.001 vs. normoxia-cultured cells from young mice; ††P<0.01 and †††P<0.001 vs. normoxia-cultured cells from aged mice; ###P<0.001 vs. hypoxia-preconditioned cells from young mice (n=3 or 4). NS, not significant.

described previously.[12,13,30] The recovery of perfusion in the ischemic hindlimb of each mouse was estimated by the percentage of limb blood flow, which was calculated by the average perfusion of the left hindlimb compared with that of the normal right hindlimb.

Statistical Analysis

All data are expressed as means±SD. Differences between mean values of multiple groups were evaluated by 1-way ANOVA followed by Scheffe's procedure. Comparisons between 2 groups were made using an unpaired Student's t-test.

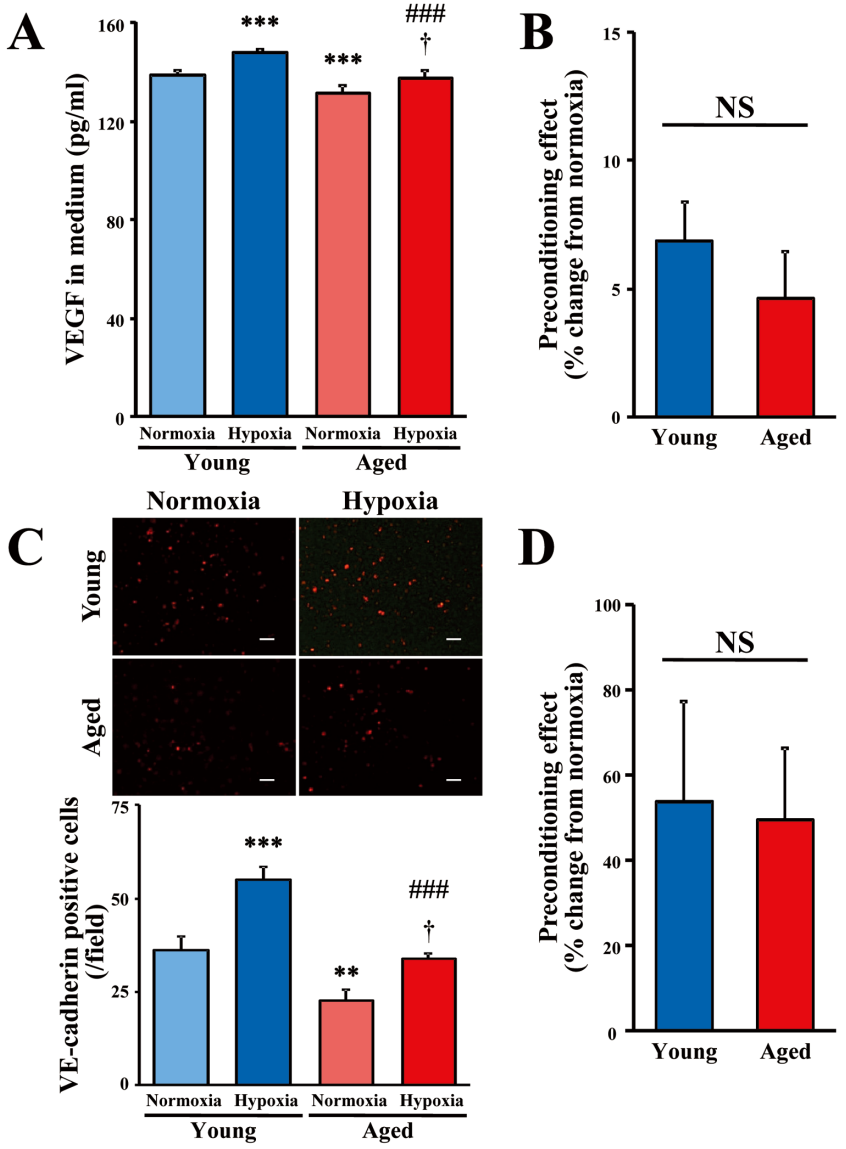

Figure 4. Effects of hypoxic preconditioning on the production of vascular endothelial growth factor (VEGF) and endothelial differentiation in bone marrow cells (BMCs). Hypoxia-preconditioned and normoxia-cultured cells from young and aged mice were cultured, and angiogenic potency was assessed. (**A**,**B**) VEGF concentration in the medium 3 days after culture. The production of VEGF was higher in the hypoxia-preconditioned cells than in the normoxia-cultured cells (**A**), and the preconditioning effect was similar between young and aged mice (**B**). (**C**,**D**) Endothelial differentiation of BMCs 7 days after culture. Representative images of VE-cadherin positive cells (red) are shown (**C**, **Upper images**). Scale bars: 50 μm. Quantitative analysis indicated that the number of VE-cadherin positive cells was higher among hypoxia-preconditioned cells than among normoxia-cultured cells (**C**, **Lower graph**), and the preconditioning effect in aged mice was comparable to that of young mice (**D**). **P<0.01 and ***P<0.001 vs. normoxia-cultured cells from young mice; †P<0.05 vs. normoxia-cultured cells from aged mice; ###P<0.001 vs. hypoxia-preconditioned cells from young mice (n=3 or 4). NS, not significant.

A value of P<0.05 was considered significant. All analyses were performed with StatView software (Version 5.0; Abacus Concepts, Calabosus, CA, USA).

Results

Aging-Related Impairment of BMCs

To characterize and determine the differences in BMCs between aged and young mice, we examined the expression of CD34 and c-kit in freshly isolated BMCs. Flow cytometry analysis showed that the percentage of cells positive for CD34 and c-kit was significantly lower in the BMCs from aged mice than in those from young mice (P<0.01, **Figures 1A**,**B**). Moreover, cell senescence in BMCs was evaluated by the expression of p53 and telomerase activity. The expression of p53 (P<0.001, **Figure 1C**) was significantly higher, and telomerase activity (P<0.05, **Figure 1D**) was significantly lower in the BMCs from aged mice than in those from young mice. These results suggest an aging-related impairment of BMCs.

Hypoxic Preconditioning and the Viability of BMCs

We examined cell survival after 24 h under hypoxic or normoxic conditions. After 24 h of exposure to 2% O_2 conditions at 33°C, the viability of the BMCs was maintained at approximately 90% (**Figure 2A**), and the number of the cells was almost the same as the number of freshly isolated BMCs seeded for hypoxic preconditioning. Compared with hypoxia-preconditioned cells, cell survival was lower in the normoxia-cultured cells. In agreement with these results, there were more apoptotic cells among the normoxia-cultured cells than among the hypoxia-preconditioned cells (P<0.05) (**Figure 2B**). There was no significant difference in cell survival and apoptosis between aged and young mice.

Hypoxic Preconditioning of BMCs and Adhesion Capacity and Resistance to Oxidative Stress

We examined the effect of hypoxic preconditioning on the capacity of BMCs to adhere to fibronectin-coated plates. Significantly fewer adherent cells were present in the normoxia-exposed cells from aged mice than in those from young mice

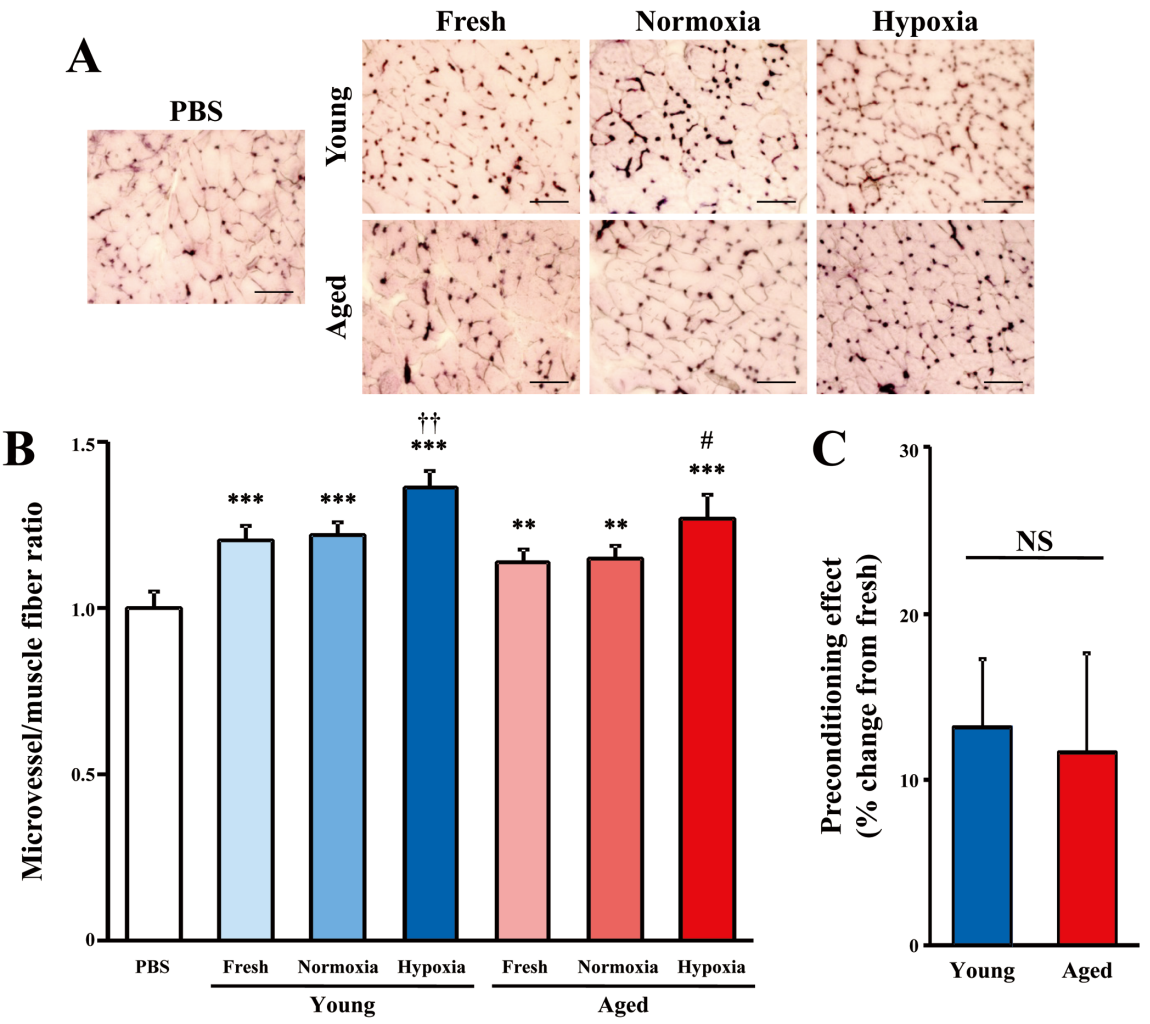

Figure 5. Histological analysis of microvessel density in the ischemic hindlimbs. (**A**) Representative images of microvessels 14 days after treatment. Scale bars: 100 μm. (**B**,**C**) Quantitative analysis revealed that the implantation of the hypoxia-preconditioned cells increased the density of microvessels in the ischemic limbs compared with normoxia-cultured cells (**B**) and that the preconditioning effect was similar between young and aged mice (**C**). **P<0.01 and ***P<0.001 vs. the mice injected with phosphate-buffered saline; ††P<0.01 vs. the mice implanted with freshly isolated and normoxia-cultured cells from young mice; #P<0.05 vs. the mice implanted with freshly isolated and normoxia-cultured cells from aged mice (n=4 or 5). NS, not significant.

(P<0.05) (**Figure 3A**), suggesting an aging-related functional impairment in adherence for BMCs from aged mice. Interestingly, compared with the normoxia-cultured cells, hypoxic preconditioning of cells from aged mice increased the adhesion capacity (P<0.01), although there was a lower tendency as compared with the hypoxia-preconditioned cells from young mice (P=0.064).

To further examine whether the hypoxia-preconditioned BMCs were resistant to oxidative stress, we assessed cell survival after exposure to H_2O_2, a powerful oxidant. Under oxidative stress, the survival rate of the normoxia-cultured cells from aged mice was significantly lower than that of the normoxia-cultured cells from young mice (P<0.001) (**Figure 3C**). More importantly, the survival rate was significantly higher in the hypoxia-preconditioned cells from aged mice than in the normoxia-cultured cells from aged mice (P<0.001), although that of hypoxia-preconditioned cells differed significantly between BMCs from aged and young mice (P<0.001).

The hypoxic preconditioning effects on cell adhesion capacity (P=0.37) (**Figure 3B**) and resistance to oxidative stress (P=0.79) (**Figure 3D**) of BMCs were found to be similar between aged and young mice.

Hypoxic Preconditioning and the Release of VEGF and Endothelial Differentiation From BMCs

We assessed the effect of hypoxic preconditioning on the angiogenic potency of BMCs. The concentration of VEGF in the supernatant 3 days after culture was significantly lower in the normoxia-cultured cells from aged mice than in those from young mice (P<0.001) (**Figure 4A**). However, the concentration of VEGF in the supernatant was significantly higher in the hypoxia-preconditioned cells from aged mice than in normoxia-cultured cells (P<0.05).

After 7 days of culture, endothelial differentiation was evaluated by immunostaining for VE-cadherin. The number of VE-cadherin-positive cells was significantly lower in the nor-

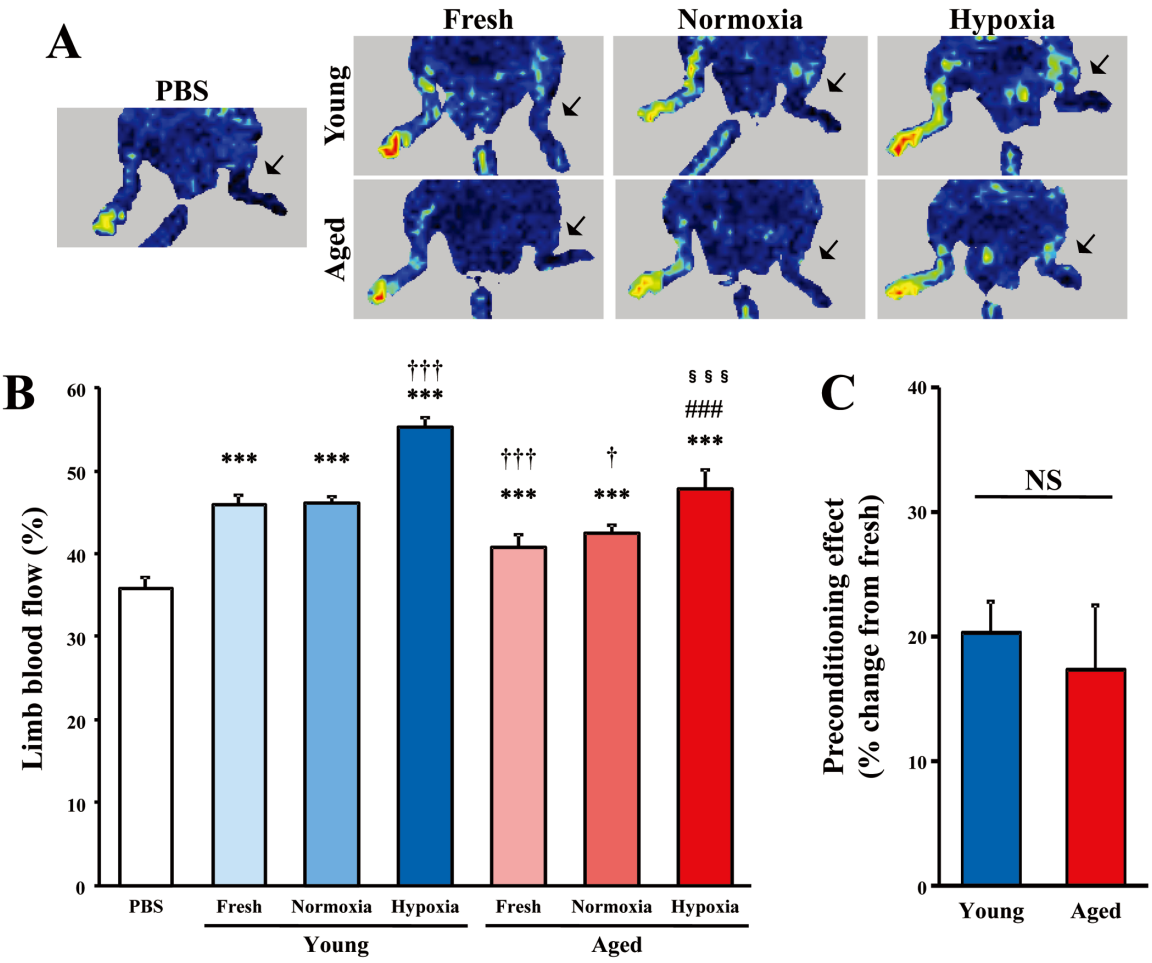

Figure 6. Blood flow recovery in the ischemic hindlimb. (**A**) Representative color-coded images representing blood flow distribution 14 days after treatment. Arrows indicate the ischemic hindlimbs. (**B**,**C**) Quantitative analysis showed that the implantation of hypoxia-preconditioned cells improved perfusion in the ischemic hindlimbs compared with the normoxia-cultured cells (**B**) and that the preconditioning effect was comparable between young and aged mice (**C**). ***P<0.001 vs. the mice injected with phosphate-buffered saline; †P<0.05 and †††P<0.001 vs. the mice implanted with freshly isolated and normoxia-cultured cells from young mice; ###P<0.001 vs. the mice implanted with freshly isolated and normoxia-cultured cells from aged mice; §§§P<0.001 vs. the mice implanted with hypoxia-preconditioned cells from young mice (n=5 or 6). NS, not significant.

moxia-cultured cells from aged mice than in those from young mice (P<0.01) (**Figure 4C**). Interestingly, significantly more VE-cadherin positive cells were observed in the hypoxia-preconditioned cells from aged mice than in the normoxia-cultured cells from aged mice (P<0.05).

Given the significant increase in the production of VEGF and endothelial differentiation, the angiogenic potency of BMCs from aged mice may be enhanced after hypoxic preconditioning. Furthermore, there was no significant difference in the preconditioning effect on VEGF production (P=0.14) (**Figure 4B**) or endothelial differentiation (P=0.81) (**Figure 4D**) between BMCs from aged and young mice, although there were significant differences between the hypoxia-preconditioned BMCs from aged mice and those from young mice (P<0.001).

Hypoxic Preconditioning and the Ability of BMCs to Induce Therapeutic Angiogenesis in an Ischemic Hindlimb Murine Model

To evaluate whether hypoxic preconditioning of BMCs enhances their therapeutic potential in vivo, we implanted BMCs into the ischemic hindlimb of aged mice. Microvessel density, an index of neovascularization, was examined by staining for alkaline phosphatase, 14 days after treatment (**Figure 5A**). Interestingly, microvessel density in the ischemic limbs implanted with hypoxia-preconditioned cells from aged mice was significantly higher than in those implanted with freshly isolated or normoxia-cultured cells (P<0.05) (**Figure 5B**), which was similar to the preconditioning effect observed in BMCs from young mice (P=0.66) (**Figure 5C**). Microvessel density in the ischemic limbs implanted with hypoxia-preconditioned cells did not significantly differ between cells from young and aged mice (P=0.22).

Perfusion of the ischemic hindlimbs was evaluated 14 days after treatment (**Figure 6A**). Although the same number of cells was delivered into the ischemic limbs, the blood flow was significantly lower in the mice implanted with freshly isolated or normoxia-cultured cells from aged mice than in those implanted with freshly isolated or normoxia-cultured cells

from young mice (P<0.05) (Figure 6B). Importantly however, blood flow in the ischemic limbs implanted with hypoxia-preconditioned cells from aged mice was significantly higher than in those implanted with freshly isolated or normoxia-cultured cells from aged mice (P<0.001); a similar preconditioning effect was observed in BMCs from young mice (P=0.25) (Figure 6C), although blood flow in the ischemic limbs was also observed to be significantly higher in mice implanted with hypoxia-preconditioned BMCs from aged mice than those from young mice (P<0.001).

Discussion

The data from the present study demonstrate that hypoxic preconditioning enhanced the in vitro function of BMCs from aged mice. We also found that the implantation of hypoxia-preconditioned BMCs from aged mice into ischemic tissue effectively induced therapeutic angiogenesis. Furthermore, hypoxic preconditioning-induced enhancement of angiogenic potential of BMCs from aged mice followed the same trend as that of BMCs from young mice.

The main purpose of this study was to assess the response of BMCs from aged mice to hypoxic pretreatment, because the effect of hypoxic pretreatment on cells from an aged donor is unknown. Consistent with previous observations of age-related impairment of cell function,[19–25] we found a decreased number of immature stem/progenitor cells, accelerated cell senescence, and impaired ability to induce therapeutic angiogenesis in BMCs from aged mice. Interestingly, hypoxic preconditioning enhanced the in vitro function of BMCs from aged mice. Furthermore, when implanted into ischemic hindlimbs, hypoxia-preconditioned BMCs from aged mice induced greater therapeutic angiogenesis. More importantly, hypoxic preconditioning can enhance the function and therapeutic potential of BMCs from aged mice in a manner similar to those from young mice.

Using BMCs from young healthy animals, our series of previous studies has demonstrated that hypoxic preconditioning increases the angiogenic potential of BMCs by enhancing the cell retention/survival and the secretion of angiogenic factors after implantation into the ischemic tissue.[11–13] In the present study, we found a similar preconditioning effect in the BMCs from both aged and young mice. Although we did not investigate in detail in this study, we believe that the mechanisms of hypoxic preconditioning in aged BMCs would be similar to those discovered in previous studies using young healthy BMCs.

It has been found that culture in low oxygen (hypoxia) can improve the stemness of stem cells.[31,32] The increased number of VE-cadherin-positive cells among hypoxia-preconditioned BMCs suggests improved endothelial commitment/differentiation of BMCs. Considering the low-oxygen microenvironment of stem cells,[33] hypoxia preconditioning may be favorable for survival and differentiation of immature stem/progenitor cells, although further experiments are required to address this issue.

This present study provides clear evidence that short-term (24 h) ex vivo hypoxic preconditioning enhanced the therapeutic efficacy of BMCs from aged as well as young mice, in addition to the fact that the number of viable cells after 24 h of hypoxic preconditioning was similar to that of (seeded) freshly isolated cells. Hypoxic preconditioning has a beneficial effect similar to that achieved by gene modification or ex vivo long-term expansion,[5–10] but might also have the advantages of efficiency, simplicity, and safety. Meanwhile, preconditioning with one or more growth factors has been shown to enhance the therapeutic efficacy of BMCs.[34–36] Given that hypoxic preconditioning induces the expression of various proangiogenic growth factors, including VEGF, basic fibroblast growth factor, stromal cell-derived factor-1 and erythropoietin,[11,15,16,37] we think that the enhancement of therapeutic potential by hypoxic preconditioning could be a synergistic effect resulting from upregulation of multiple growth factors in culture under hypoxic conditions. From a clinical perspective, we believe that ex vivo hypoxic preconditioning may be an effective, simple and safe strategy by which to increase the therapeutic efficiency of cell-based angiogenesis.

Similar to previous studies that used cells from a healthy young donor, hypoxic preconditioning was demonstrated to enhance the therapeutic potential of BMCs to induce angiogenesis in cells with an aging-related functional impairment. Although further studies may be needed to confirm the positive effects of hypoxic preconditioning using BMCs from individuals with systemic complications such as diabetes mellitus, we conclude that hypoxic preconditioning of donor cells before implantation is a simple and feasible strategy for improving the therapeutic effectiveness of cell-based angiogenesis in future clinical trials.

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