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Hypoxic preconditioning reinforces cellular functions of autologous peripheral blood-derived cells in rabbit hindlimb ischemia model

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ABSTRACT

Peripheral blood mononuclear cell (PBMNC) is one of powerful tools for therapeutic angiogenesis in hindlimb ischemia. However, traditional approaches with transplanted PBMNCs show poor therapeutic effects in severe ischemia patients. In this study, we used autograft models to determine whether hypoxic pretreatment effectively enhances the cellular functions of PBMNCs and improves hindlimb ischemia. Rabbit PBMNCs were cultured in the hypoxic condition. After pretreatment, cell adhesion, stress resistance, and expression of angiogenic factor were evaluated *in vitro*. To examine *in vivo* effects, we autografted preconditioned PBMNCs into a rabbit hindlimb ischemia model on postoperative day (POD) 7. Preconditioned PBMNCs displayed significantly enhanced functional capacities in resistance to oxidative stress, cell viability, and production of vascular endothelial growth factor. In addition, autologous transplantation of preconditioned PBMNCs significantly induced new vessels and improved limb blood flow. Importantly, preconditioned PBMNCs can accelerate vessel formation despite transplantation on POD 7, whereas untreated PBMNCs showed poor vascularization. Our study demonstrated that hypoxic preconditioning of PBMNCs is a feasible approach for increasing the retention of transplanted cells and enhancing therapeutic angiogenesis in ischemic tissue.

1. Introduction

Coronary artery disease, cerebrovascular disease and peripheral arterial disease are associated with substantial mortality, and the morbidity of these ischemic diseases increases with age and lifestyle-related illness such as hypertension, diabetes, and dyslipidemia [1]. The prognosis of severe limb ischemia patients with rest pain, ulcers, and gangrene is poor, and traditional therapeutic treatments such as angioplasty and surgery are ineffective in many of these patients [2]. Therapeutic angiogenesis, which can be induced by cell transplantation and growth factor delivery to ischemic areas, is among the most beneficial therapeutic approaches to vascular diseases [3]. In this approach, the therapeutic availability of both bone marrow- and peripheral blood-derived cells has been examined in animal models and human clinical trials owing to their easy preparation and maintenance [4–6]. However, the effects of cell-based therapeutic angiogenesis are prohibited by ischemic conditions in tissue.

Ischemia causes hypoxia by interrupting red blood cell influx and causing up-regulation of inflammatory cytokines, resulting in excessive production of reactive oxygen species (ROS) in ischemic tissue [7–9]. Accumulation of ROS destroys transplanted cells through induction of apoptosis and necrosis [10], which were believed to be factors in the poor retention of these cells in ischemic area [11]. To overcome these challenges, researchers have tested several protocols, such as genetic modification of transplant cells that have increased of retention [12,13]. However, current protocols using genetic modification or cell sorting to concentrate specific cell types limit clinical applications. Cost, ethical concern, and time-consuming processes must be minimized in applications for therapeutic angiogenesis. We recently developed a novel and feasible protocol called "hypoxic preconditioning", to enhance the cellular functions of transplant cells and improve retention of transplanted cells. Hypoxically pretreated mouse bone marrow cells or peripheral blood mononuclear cells (PBMNCs) survived in ischemic tissues, and therapeutic angiogenesis was induced at high

Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction; CXCR4, C-X-C chemokine receptor type 4; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ROS, reactive oxygen species; DCF, 6-carboxyl-2′,7′-dichlorodihydrofluorescein; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; PBMNC, peripheral blood mononuclear cell.

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level than that achieved with untreated cells in a mouse model of hindlimb ischemia [14,15]. The effect of hypoxic preconditioning are also observed in bone marrow-derived cells including mesenchymal stem cells, and hypoxic preconditioning increases cell adhesion, migration and survival as well as PBMNCs [16,17]. Although precise molecular mechanisms are not fully understood, up-regulation of CXCR4, c-Met, pAkt and/or HIF-1α expression by hypoxic treatment is thought to induce reinforcement of these cellular functions. Our previous study showed that SDF-1, a ligand for CXCR4, is up-regulated in the muscle of ischemic hindlimb, suggesting longer retention of pre-conditioned cells in ischemic area [18]. Therefore, we believe that hypoxic preconditioning is a powerful tool for improving therapeutic angiogenesis in severe ischemia patients.

In this study, we performed pre-clinical testing of hypoxic preconditioning for future human trials using a rabbit model of ischemia. Autologous rabbit PBMNCs (rPBMNCs) were grafted into ischemic hindlimbs after hypoxic pretreatment to examine their contributions to therapeutic angiogenesis *in vivo*. Angiogenesis in ischemic hindlimb was enhanced by autotransplantation of hypoxically pretreated rPBMNCs, and newly developed microvessels were functionally recovered. The efficacy of therapeutic angiogenesis with pretreated rPBMNCs was better than that with untreated cells. A series of *in vitro* and *in vivo* experiments demonstrated that hypoxic preconditioning of PBMNCs is a viable protocol for not only small animals but also larger animals.

2. Materials and methods

2.1. Ethics statement

All experiments using human subjects were approved by the Medical Ethics Committee of Yamaguchi University School of Medicine (MECYUSM; No. H23-44-4). Informed consent to collect blood samples was written and obtained from enrolled all patients according to the MECYUSM guidance.

2.2. Animals

Male New Zealand white rabbits (3.0–3.5 kg body weight, KBT Oriental, Tosu, Saga, Japan) were used for the animal experiments. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC; No. 31-084) of Yamaguchi University. The study was conducted in accordance with the Declaration of Helsinki.

2.3. Isolation and hypoxic preconditioning of PBMNCs

PBMNCs (rPBMNCs) were isolated from rabbit peripheral blood as described previously [8]. rPBMNCs were preconditioned in the culture at 33 °C in 2% O_2 and 5% CO_2 for 24 h (hypoxia), as described previously [7]. rPBMNCs cultured under normoxic conditions (33 °C in 20% O_2 and 5% CO_2 for 24 h) were used as a control (normoxia).

2.4. Cell adhesion assay

After 24 h of culture under normoxic or hypoxic conditions, rPBMNCs (2×10^6/ml per well) were plated onto fibronectin-coated 24-well culture plates and cultivated under normal cell culture conditions. After 24 h, the wells were immersed in 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution for 10 min to visualize cell nuclei. The number of attached cells was counted in 5 randomly chosen microscopic fields (200× magnification) per well. Data are expressed as the number of cells per field.

2.5. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

To examine the effect of hypoxic preconditioning on CXC chemokine receptor 4 gene (*cxcr4*) expression, we performed semi-quantitative RT-PCR. Specific primers were designed as follows: *cxcr4*, forward 5′-GGTGGTCTACGTCGGTGTCT-3′ and reverse 5′-TGGAGTGTGACAGCTTGAG-3′; glyceraldehyde 3-phosphate dehydrogenase (*gapdh*), forward 5′-CGCCTGGAGAAAG CTGCTAA-3′ and reverse 5′-CGACCTGGTCCTCGGTGTAG-3′. Band intensity was quantified using ImageJ software, and the *cxcr4* expression level was normalized to that of *gapdh*.

2.6. Assay for oxidative stress resistance

To examine whether hypoxic preconditioning affects the tolerance of PBMNCs for oxidative stress, we exposed hypoxically or normoxically cultured rPBMNCs to growth medium including 100 μM H_2O_2 for 24 h. Intracellular reactive oxygen species (ROS) were measured using a 6-carboxyl-2′,7′-dichlorodihydrofluorescein diacetate (DCF) probe (Lambda Fluorescence Technology, Graz, Austria) as described previously [7]. Data are expressed as the percentages of DCF fluorescence of hypoxic-preconditioned PBMNCs to that of normoxic-cultured PBMNCs.

2.7. Apoptosis and cell viability assay

Apoptosis was analyzed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA). Oxidative stress was induced by the addition of 100 μM H_2O_2 to both preconditioned and normally cultured cells for 24 h. Annexin V-expressing cells and propidium iodide (PI)-incorporated cells were analyzed with flow cytometry. Live cells were evaluated as Annexin-negative and PI-negative cells.

2.8. Hindlimb ischemic model and cell transplantation

Rabbits were anesthetized, and the left femoral artery, popliteal artery, and its branches in the left hindlimbs were removed to induce ischemia. On postoperative day (POD) 6, rPBMNCs were collected and cultured under hypoxic or normoxic conditions for 24 h. And then, rPBMNCs labeled with a red fluorescent dye (PKH26; Sigma, St. Louis, MO, USA) were injected intramuscularly into ischemic regions (6 points with 10 μl PBS or 1×10^7 cells per point). Rabbits were divided into the following 4 groups: PBS (injection of PBS, $n = 6$); fresh (injection of isolated rPBMNCs, $n = 6$; normoxia (injection of normally-cultured rPBMNCs, $n = 6$; and hypoxia (injection of hypoxically preconditioned rPBMNCs, $n = 6$).

2.9. Measurement of blood flow in ischemic hindlimbs

Blood flow in ischemic hindlimb was measured using a laser Doppler perfusion imaging system (PeriScan System; Perimed AB, Stockholm, Sweden) at preoperation, POD 0 (just after operation), and POD 3, 7, 14, 21, and 28. Both intact (right) and ischemic (left) hindlimbs were scanned, and mean perfusion scores were obtained from each. The recovery of perfusion in ischemic hindlimbs was evaluated by determining the percentage of blood flow expressed as the average perfusion score in the left hindlimb normalized by that in the right ($n = 6$). All procedures were performed under slight anesthesia.

2.10. Evaluation of blood flow recovery in ischemic hindlimbs

On POD 28, eosin Dye-Trak microspheres (6×10^5 beads/200 μl; Triton Technology, Loughborough, UK) were injected into the abdominal aorta under anesthesia. Rabbits were sacrificed after 30 s of injection, and skeletal muscles in the ischemic area were collected from the hindlimb. To collect the microspheres, we completely digested the muscles in 16 N KOH for 48 h at 60 °C. After extracting the fluorescent dye from the microspheres with dimethylformamide, we measured the optical density (OD) with a spectrophotometer (PerkinElmer, Waltham, MA, USA). Recovery of perfusion in the ischemic hindlimb was evaluated by the score calculated as (ischemic hindlimb OD/intact hindlimb OD) × (intact hindlimb tissue weight/ischemic hindlimb tissue weight) ($n = 4$) [8].

2.11. Microvessel density

On POD 28, the quadriceps and adductor muscles were removed. To visualize microvessels, we stained frozen sections (7-μm thickness) of the ischemic muscle for alkaline phosphatase with an indoxyl tetrazolium method [7]. The number of microvessels and muscle fibers were counted in 20 randomly chosen fields (200× magnification). Microvessel density was evaluated by examining microvessel/muscle fiber ($n = 4$).

2.12. Statistical analysis

All data are expressed as means ± standard error. Differences between mean values of multiple groups were evaluated with analysis of variance followed by Scheffe's procedure. Comparisons between 2 groups were made with the unpaired Student's *t*-test. A *P* value of <0.05 was considered significant. All analyses were performed with the SPSS software (IBM, Chicago, IL, USA).

3. Results

3.1. Functional reinforcement of rPBMNCs via hypoxic preconditioning

To examine the effect of hypoxic preconditioning on the adherent capacity of rPBMNCs, we counted the number of attached cells on the cell culture dish 24 h after plating. The number of attached cells was significantly increased in the hypoxia group ($P < 0.01$: Fig. 1A). In addition, CXCR4 gene expression was up-regulated in the preconditioned rPBMNCs ($P < 0.05$: Fig. 1B).

We next examined functional augmentation of rPBMNCs in resistance to oxidative stress through hypoxic preconditioning. rPBMNCs were cultured under hypoxic or normoxic conditions with H_2O_2, and intracellular ROS level was measured after 24 h of incubation. ROS level in preconditioned cells (hypoxia) was significantly lower than that in normoxically cultured cells (normoxia) ($P < 0.05$: Fig. 2A), which increased cell survival under oxidative stress conditions ($P < 0.05$: Fig. 2C). By contrast, both ROS accumulation and cell survival after incubation without H_2O_2 were not significantly different between the hypoxia and the normoxia conditions (Fig. 2B and D). Interestingly, apoptotic cell number was not altered in either group (Fig. 2E and F). Also, hypoxic preconditioning increased VEGF secretion in human PBMNCs (Supplementary Fig. 1). Taken together, hypoxic preconditioning reinforced cellular functions for cell adhesion, cell mobilization, and resistance to oxidative stress of rPBMNCs consistent with the results of small animal studies [7,8].

3.2. Improvement of ischemia with autologous transplantation of preconditioned PBMNCs

To examine whether hypoxically preconditioned rPBMNCs improve hindlimb ischemia, we grafted autologous rPBMNCs into ischemic hindlimbs after pretreatment. On postoperative day (POD) 6, rPBMNCs were isolated and preconditioned in hypoxic culture condition for 24 h. After labeling with red fluorescent dye (PKH26), the preconditioned rPBMNCs were grafted into ischemic areas. At POD 28, a small number of preconditioned rPBMNCs remained in the skeletal muscle in the ischemic areas (Fig. 3A). Perfusion of ischemic hindlimbs was evaluated at preoperation, POD 0 (just after operation), and PODs 3, 7, 14, 21, and 28 using a laser Doppler perfusion imaging system. Among all experimental groups, the hypoxia group showed significantly increased blood flow in ischemic limbs (Fig. 3B and C). In addition, preconditioned rPBMNCs led to approximately half recovery of blood flow (46.6 ± 5.8%) at POD 28, and the recovery rate after surgery was

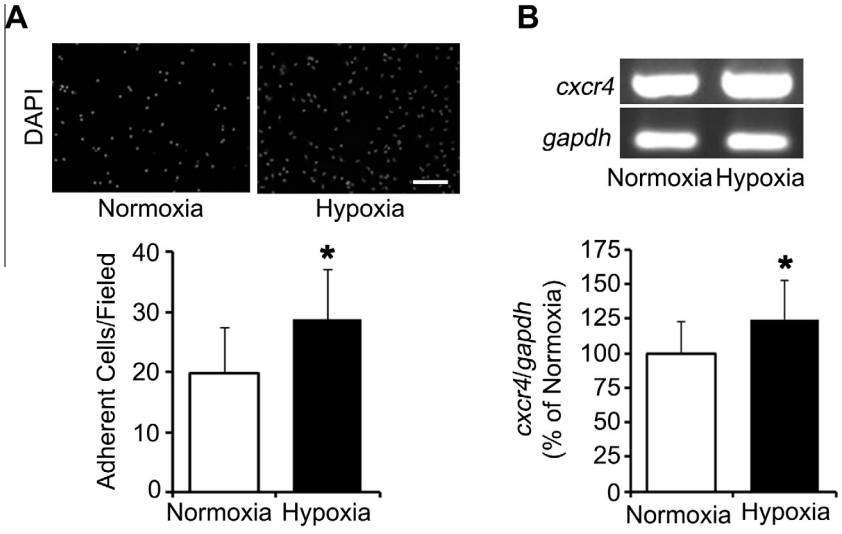

Fig. 1. Hypoxic preconditioning enhances cell adhesion and chemokine receptor expression in rabbit PBMNCs. rPBMNCs were cultured under normoxic (Normoxia) and hypoxic (Hypoxia) conditions for 24 h. Then preconditioned and normally cultured rPBMNCs were plated on a laminin-coated dish. (A) Twenty-four hours after plating, cell nuclei were visualized using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI). Attached cell number was counted to check whether hypoxic preconditioning enhanced the adhesion capacity of rPBMNCs. $n = 9$, $^*P < 0.01$ *vs.* Normoxia. Scale bar represents 200 μm. (B) To check the expression of cell mobilization factors, we collected rPBMNCs after hypoxic pretreatment and performed semi-quantitative reverse transcription-polymerase chain reaction. Hypoxic preconditioning significantly up-regulated the CXC chemokine receptor 4 gene (*cxcr4*) in rPBMNCs. $n = 9$, $^*P < 0.05$ *vs.* Normoxia.

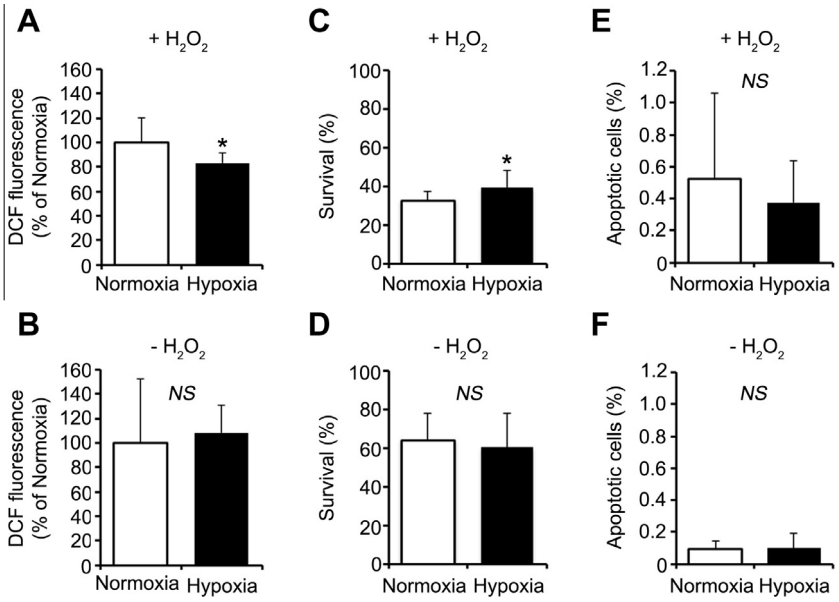

Fig. 2. Hypoxic preconditioning makes rPBMNCs resistant to oxidative stress. To check stress resistance of preconditioned PBMNCs, we exposed pretreated (Hypoxia) or untreated (Normoxia) cells to medium including 100 μM H_2O_2. Intracellular accumulation of reactive oxygen species (ROS) (A and B), cell survival (C and D) and apoptosis (E and F) under ischemic stress conditions were evaluated. ROS level was calculated as fluorescent intensity of a 6-carboxyl-2′,7′-dichlorodihydrofluorescein diacetate (DCF) probe, and live cells (survival rate) were evaluated as Annexin V^-/PI^- cells. $n = 9$, $^*P < 0.05$ *vs.* Normoxia. PI, propidium iodide. *NS*: no significance.

significantly higher in the hypoxia group than that in the other groups ($P < 0.05$: Fig. 3D).

3.3. Preconditioned rPBMNCs accelerate neovascularization in ischemic hindlimbs

Next, we examined whether PBMNC autografts enhance neovascularization in ischemic hindlimbs. Blood vessels were counted in muscle cross-sections of POD 28 samples and microvessel density was calculated. Microvessel density was significantly increased in the hypoxia group compared with that in other groups ($P < 0.05$), whereas no significant difference was found among the normoxia, fresh, and PBS groups (Fig. 4A and B). To clarify whether newly formed microvessels were functional after transplantation, we injected fluorescent labeled-microspheres—which we expected to stack in the middle of newly formed microvessels in the ischemic area—into the abdominal aorta. After the microspheres collected in the ischemic area, we calculated blood flow recovery using fluorescence intensity. Quantitative analysis showed that blood flow in the hypoxia group was higher than that in other groups ($P < 0.05$: Fig. 4C), whereas no significant difference was observed among the normoxia, fresh, and PBS groups. These results clearly showed that hypoxically preconditioned PBMNCs contribute to therapeutic angiogenesis by efficiently developing functional vessels in ischemic areas.

4. Discussion

Both PBMNCs and bone marrow-derived cells are effective for therapeutic angiogenesis in hindlimb ischemia and have promising clinical advantages, even though conclusions about which cell type is better remain controversial [19–21]. However, the efficacy of cell-based therapies varies among ischemic patients, and one of reasons for poor therapeutic efficiency is the environmental variation in ischemic tissues. Oxygen level is lower and inflammatory cytokines are relatively high in ischemic tissue, resulting in poor outcomes in therapeutic angiogenesis owing to unsuitable conditions for the survival of transplanted cells [11,22]. Furthermore, both oxygen and cytokine levels in tissues are altered in patients with complex health histories that include conditions such as diabetes [23,24]. In this study, we demonstrated that cellular functions can be reinforced in PBMNCs through hypoxic preconditioning and that preconditioned PBMNCs improved the outcome of therapeutic angiogenesis in an autograft model. In particularly, enhancement of oxidative stress resistance is expected to increase the survival of transplants in ischemic tissue, suggesting that this protocol will improve the survival of patient-derived autologous PBMNCs in ischemic limbs. Therefore, this protocol might increase the success rate of therapeutic angiogenesis in patients with ischemic disease.

The effects of hypoxic preconditioning cover multi-cellular events. In this study, hypoxic preconditioning enhanced cell retention and angiogenic potential as well as stress resistance in rPBMNCs. Although this study did not assess in detail the significance of individual reinforced functions on therapeutic angiogenesis, these multi-cellular functions are likely to act cooperatively in recovery from ischemia after transplantation, as suggested by our previous studies [7,8,14].

Autologous transplantation of preconditioned PBMNCs induced higher blood flow recovery and angiogenesis than those in other experimental groups. This result clearly indicates that hypoxic preconditioning is a better protocol for inducing effective therapeutic angiogenesis than traditional approaches using crude PBMNCs. Conversely, we also found discrepant results in microvessel density. Previous studies have demonstrated an increase in both microvessels and reperfusion after transplantation of crude PBMNCs [7,18,20,25], whereas in our study, transplantation of crude PBMNCs unexpectedly resulted in no significant increase in microvessel density despite recovered perfusion. Although the reasons for the difference in therapeutic effects between ours and other methods are unclear at this time, we think that the timing of cell transplantation after surgery is a possibility. Furthermore, this difference might result in immature capillary networks after crude PBMNC transplantation. In our protocol, PBMNCs were transplanted into ischemic hindlimbs on POD 7, whereas cells were transplanted within a few days after surgery in other protocols [7,8]. If our speculation is correct, earlier transplantation of

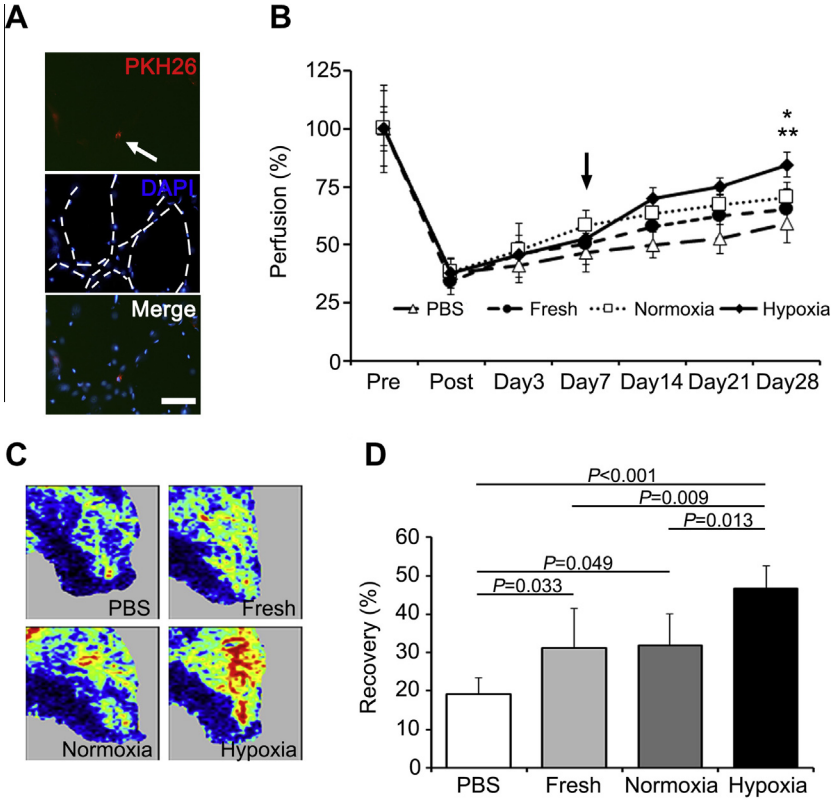

Fig. 3. Reinforced autologous PBMNCs improve blood flow in ischemic limbs. To test whether preconditioned PBMNCs improve ischemia, we pretreated autologous rPBMNCs hypoxically and grafted them into ischemic hindlimbs. Autologous PBMNCs were collected on postoperative day (POD) 6 and transplanted into ischemic hindlimb on POD 7. As control groups, phosphate-buffered saline (PBS), freshly isolated PBMNCs (Fresh), and normoxically cultured PBMNCs (Normoxia) were injected into ischemic hindlimbs. (A) To check retention of transplanted PBMNCs, we pre-stained cells with red fluorescent dye (PKH26). Few autografted rPBMNCs ($PKH26^+$) remained in the ischemic area on POD 28 (arrow). Pre, preoperation; Post, postoperation. Scale bar represents 50 μm. (B) Postsurgical perfusion was evaluated using a laser Doppler perfusion imaging system. Graph indicates the change in limb blood flow. Arrow: PBMNC transplantation. $^*P < 0.05$ *vs.* Normoxia. $^{**}P < 0.001$ *vs.* Fresh and PBS groups. (C) Representative laser Doppler images at POD 28. (D) Recovery rate (%) after cell transplantation. $n = 6$ animals/group.

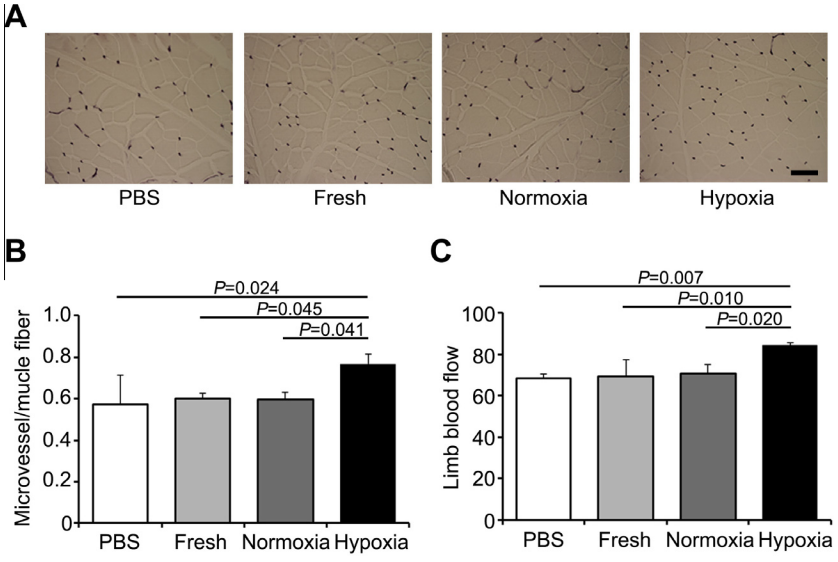

Fig. 4. Reinforced autologous PBMNCs accelerate neovascularization. Skeletal muscles were collected on POD 28, and microvessel density in ischemic areas was evaluated after cell transplantation. (A and B) Representative images of vessels in muscle cross-sections. To visualize vessels, we performed alkaline phosphatase staining (dark blue dots) on frozen muscle sections (7-μm thickness). Microvessel density in the ischemic limbs was significantly higher in the hypoxia group (lower graph). (C) To test the maturity of newly formed vessels, we injected fluorescent microspheres into the abdominal aorta on POD 28. Skeletal muscle was dissected immediately after microsphere injection and then digested to collect microspheres in microvessels. Microsphere injection demonstrated functional recovery of newly formed vessels in the hypoxia group. $n = 4$ animals/group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

preconditioned PBMNCs might increase their effects on therapeutic angiogenesis in ischemic hindlimbs, expanding clinical applications of hypoxic preconditioning. However, we think that our protocol provides the appropriate timing for the transplantation of cells exhibiting enhanced capabilities such as VEGF secretion, because the expression of VEGF receptor in vasculatures peaks at the middle stage (~day 9) of new postsurgical capillary formation in mice [26], suggesting that VEGF gives full play to cell effects at later stages of vascular regeneration but not during early stages. In addition, cell-based therapy for limb ischemia is generally expected to be used in chronic cases, and small capillary networks are thought to be formed already in ischemic areas in these patients. Although no animal model for hindlimb ischemia completely reflects the disease condition, we at least need to consider the timing of cell transplantation in preclinical studies. Therefore, PBMNCs were grafted when blood flow was naturally and gradually recovered to mimic "sub-chronic hindlimb ischemia" in our experiments.

In this study, we clearly showed that the hypoxic preconditioning protocol can be applied to PBMNC autografts and that it improves hindlimb ischemia. Preconditioned PBMNCs do not contribute to tumor development after transplantation (unpublished data), indicating that our next step is to apply this protocol to early phase human trials. Because some patients, such as those with peripheral arterial disease (PAD) patients on dialysis, show lower response to PBMNC transplantation [25], we hope that preconditioned PBMNCs will improve severe limb ischemia in these patients and improve their condition as soon as possible.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.01.054.

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