Biochemical and Biophysical Research Communications 444 (2014) 370–375

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

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ARTICLE INFO

Article history:
Received 8 January 2014
Available online 23 January 2014

Keywords:
Peripheral blood-derived mononuclear cells
Hypoxic preconditioning
Therapeutic angiogenesis
Cell-based therapy
Pre-clinical testing

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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0006-291X/$ - see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2014.01.054

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.