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Protein profiling and angiogenic effect of hypoxia-cultured human umbilical cord blood-derived mesenchymal stem cells in hindlimb ischemia

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ABSTRACT

The aim of the present study was to investigate protein profiles of human umbilical cord blood-derived mesenchymal stem cells (hUCB-MSCs) cultured in normoxic (21% O_2) and hypoxic (1% O_2) conditions, and evaluate oxygenation effects on angiogenesis in an ischemic hindlimb mouse model using a modified ischemic scoring system. Hypoxic conditions did not change the expression of phenotypic markers and increased adipogenesis and chondrogenesis. Epidermal growth factor (EGF), transforming growth factor alpha (TGF-α), TGF-β RII, and vascular endothelial growth factor (VEGF) were upregulated in the conditioned medium of hypoxic hUCB-MSCs, which are commonly related to angiogenesis and proliferation of biological processes by Gene Ontology. In the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, significant enrichment of the phosphorylation of abelson murine leukemia viral oncogene homolog 1 (ABL1) (Phospho-Tyr204) and B-cell lymphoma-extra large (BCL-XL) (Phospho-Thr47) as anti-apoptotic pathways was observed in hypoxic hUCB-MSCs. Furthermore, hypoxic conditions induced proliferation and migration, and reduced apoptosis of hUCB-MSCs *in vitro*. Based on the results of protein antibody array, we evaluated the angiogenic effects of injecting normoxic or hypoxic hUCB-MSCs (1×10^6) into the ischemic hindlimb muscles of mice. Ischemic scores and capillary generation were significantly greater in the hypoxic hUCB-MSC injection group than in the normoxic hUCB-MSC group. Our findings demonstrate that culturing hUCB-MSCs in hypoxic conditions not only significantly enriches phosphorylation in the anti-apoptosis pathway and enhances the secretion of several angiogenic proteins from cells, but also alleviates ischemic injury of hindlimb of mice.

1. Introduction

Ischemic disease results from insufficient arterial supply to tissues of the heart (Orini et al., 2015), limbs (Stella et al., 2015), brain (Kim et al., 2015) and bowel (Dashwood et al., 2016). The global rates of disability and mortality due to ischemic diseases are pronounced. According to a meta-analysis, peripheral arterial disease affected more than 202 million people worldwide in 2010 (Fowkes et al., 2013). As angiogenesis is the main process of blood vessel generation, the maintenance and enhancement of angiogenesis is crucial for the recovery of ischemia-damaged tissue.

Cell therapies with mesenchymal stem cells (MSCs) from different sources have effectively fostered recovery from ischemia-related diseases including stroke (Nam et al., 2015), coronary artery disease (Li et al., 2015c) and peripheral arterial disease (Kim et al., 2011; Kim et al., 2006). Such therapies have been shown to promote angiogenesis through paracrine (Hu et al., 2015), immunomodulatory (Yoo et al., 2013), migration (Li et al., 2015a) and differentiation effects (Jiang et al., 2015) in pre-clinical or early clinical research (Lee et al., 2011). Although MSCs have therapeutic potential, a method of enhancing their angiogenic effects in order to improve therapeutic efficacy is lacking.

MSCs are in perivascular niches of different tissues where there are

Abbreviations: ABL, abelson murine leukemia viral oncogene homolog; BCL-XL, B-cell lymphoma-extra large; Cy, Cyanine; DAVID, Database for annotation visualization and integrated discovery; DNA-PK, DNA-activated protein kinase; 4E-BP1, eukaryotic initiation factor 4E-binding protein; EGF, epidermal growth factor; FBS, fetal bovine serum; ETK, epithelial and endothelial tyrosine kinase; FITC, fluorescein isothiocyanate; GO, Gene ontology; HCl, Hydrogen chloride; hUCB-MSC, human umbilical cord blood-derived mesenchymal stem cell; ILAR, Institute of Laboratory Animal Resources; KEGG, Kyoto Encyclopedia of Genes and Genomes; LYN, Lck/Yes novel tyrosine kinase; MSC, mesenchymal stem cell; PBS, phosphate buffered saline; PD-ECGF, platelet-derived endothelial cell growth factor; PE, phycoerythrin; PECAM, platelet endothelial cell adhesion molecule; PerCP, peridinin-chlorophyll proteins; PIGF, placental growth factor; PKC, protein kinase C; SBRI, Samsung Biomedical Research Institute; SYN1, synapsin; TGF, transforming growth factor

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low oxygen concentrations due to hypoxic conditions (Mohyeldin et al., 2010). *In vitro* hypoxic culture conditions have varying effects on different kinds of cells: for instance, these conditions promote the proliferation (Li et al., 2016) and survival (Parks et al., 2016) of tumors, improve the undifferentiated state and self-renewal of embryonic stem cells (Barbosa et al., 2012) and MSCs (Park et al., 2013) and enhance angiogenesis and growth factor secretion in endothelial cells (Liu et al., 2015a). In addition, several studies have reported that angiogenesis was enhanced when MSCs isolated from adipose (De Barros et al., 2013), bone marrow (Liu et al., 2015b) and umbilical cord (Han et al., 2016) were cultured in hypoxic conditions.

In our previous study, we investigated the gene expression of angiogenesis- and immunity-related cytokines *in vitro* and *in vivo* using hypoxic human umbilical cord blood-derived mesenchymal stem cells (hUC-MSCs) (Han et al., 2016). In this study, we compared the expression of secretory proteins and phosphorylated proteins in hypoxic and normoxic hUCB-MSCs using antibody arrays, and evaluated recovery from ischemia-induced injury with our modified ischemic scoring system in an ischemic hindlimb mouse model injected with hypoxic hUCB-MSCs.

2. Materials and methods

2.1. Culture of hUCB-MSCs

hUCB-MSCs were provided by Kangstem Biotech (Seoul, Korea) and were cultured in KSB-3 Complete Medium (Kangstem Biotech, Seoul, Korea) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and 0.1% antibiotic/antimycotic (Gibco, Carlsbad, CA, USA) at 37 °C and 5% CO_2. hUCB-MSCs were maintained until passage 3 in normoxic culture conditions, and then were divided into normoxic and hypoxic culture conditions and continuously expanded until passage 6 for *in vitro* and *in vivo* experiments. The culture duration from passage 3 to passage 6 was 2 weeks under both culture conditions. Exchanging media and passaging cells were performed in a normoxic environment. The hypoxic and normoxic culture conditions (1% O_2 and 21% O_2, respectively) were applied in a multigas incubator (APM-30D; ASTEC, Fukuoka, Japan).

2.2. Flow cytometry

hUCB-MSCs were fixed with Cytofix™ fixation buffer (BD Biosciences, Piscataway, NJ, USA), and washed twice with Stain Buffer (BD Biosciences). For the analysis of phenotype markers, hUCB-MSCs were stained with V450 mouse anti-human CD31 (1:20), fluorescein isothiocyanate (FITC) mouse anti-human CD34 (1:20), phycoerythrin (PE)-Cy™7 mouse anti-human CD44 (1:20), V500 mouse anti-human CD45 (1:20), PerCP-Cy™5.5 mouse anti-human CD73 (1:20), PE mouse anti-human CD90 (1:20) and APC mouse anti-human CD105 antibodies (1:20) (BD Biosciences). Cells were stained with a particular antibody for 30 min at room temperature, washed twice with Stain Buffer (BD Biosciences) and examined with a FACSVerse™ flow cytometer (BD Biosciences) and Flowjo software (Treestar, San Carlos, CA, USA).

2.3. Differentiation assay

Normoxic and hypoxic hUCB-MSCs at passage 6 were seeded at a concentration of 1.5×10^5 cells in a 12-well culture plate with complete medium. After overnight incubation, cells were treated with adipogenic and osteogenic differentiation medium (CEFO, Seoul, Rep. of Korea). For analysis of adipogenic differentiation, cells were washed with PBS and stained with Oil Red O for adipogenic differentiation (CEFO, Seoul, Rep. of Korea) at 28 days according to the manufacturer's instructions. For analysis of osteogenic differentiation, cells were washed with PBS, and stained with Alizarin Red for osteogenic differentiation (CEFO, Seoul, Rep. of Korea) at 21 days. Quantification of both differentiations was analyzed by measuring absorbance at 550 nm with a GLOMAX multi detection system (Promega, Sunnyvale, CA, USA). For analysis of chondrogenic differentiation, cells were seeded at a concentration of 1.5×10^5 cells in a 12-well culture plate with complete medium. After overnight incubation, attached cells were treated with chondrogenic differentiation medium (CEFO, Seoul, Rep. of Korea) and stained with Alcian blue (Sigma-Aldrich, St Louis, MO, USA) at 36 days. For quantification of chondrogenic differentiation, the spheroid culture method was conducted. 5×10^5 cells were seeded in a 15 ml tube and centrifuged at 300*g* for 3 min. After removing supernatant, the cell pellet was treated with chondrogenic differentiation medium (CEFO, Seoul, Rep. of Korea) and incubated for 36 days. After removing the differentiation medium, the weight of the generated spheroids was measured. Spheroids were transferred to 96-well plates, fixed with 4% formaldehyde (Sigma-Aldrich, St Louis, MO, USA) for 30 min, and washed with distilled water. Spheroids were stained with 1% Alcian blue at room temperature overnight, and washed with 0.1N HCl (Sigma-Aldrich, St Louis, MO, USA) and distilled water. The images of stained spheroids were acquired with a digital camera F2.5 (Samsung, Seoul, Rep. of Korea). To elute Alcian blue from the stained spheroids, 8 M Guanidine HCl solution (Sigma-Aldrich, St Louis, MO, USA) was added to the spheroids. After incubating at 4 °C overnight, supernatant from spheroids was transferred to 96-well plates for measuring absorbance of released Alcian blue at 600 nm.

2.4. Cell proliferation assay

Normoxic and hypoxic hUCB-MSCs at passage 6 were seeded at a concentration of 4×10^4 cells in a 100-mm culture plate with complete medium, and were cultured in the normoxic and hypoxic culture conditions (21% O_2 and 1% O_2, respectively). At 1, 3 and 5 days, cells were detached with 0.25% trypsin and counted following Trypan blue staining.

2.5. Cell migration assay

Normoxic and hypoxic hUCB-MSCs at passage 6 were seeded at a concentration of 3×10^5 cells in a μ-Dish 35-mm culture insert (ibidi GmbH, Munich, Germany) containing complete medium in the normoxic and hypoxic culture conditions (21% O_2 and 1% O_2, respectively). After overnight incubation, each culture insert was removed and cells were incubated for 16 h. Cell images were acquired with a model CKX41 inverted microscope (Olympus Corporation, Tokyo, Japan). After 0 or 16 h of incubation, the density percentage of cell migration (cell area of insert at 16 h was divided by non-cell area of insert at 0 h, and multiplied by 100) was measured with Infinity Analyze 6.1.0 software (Lumenera Corporation, Ottawa, ON, Canada).

2.6. Apoptosis assay

Normoxic and hypoxic-MSCs at passage 6 were seeded at a concentration of 2×10^5 cells in a 6-well culture plate containing complete medium in the normoxic and hypoxic culture conditions (21% O_2 and 1% O_2, respectively). After overnight incubation, cells were treated with 0, 0.5, 1 and 2 μM of camptothecin (Sigma-Aldrich, St Louis, MO, USA) and incubated for 48 h. Then, cells were detached with 0.25% of Trypsin-EDTA (Gibco, Grand Island, NY, USA) and stained with FITC Annexin V apoptosis Detection Kit I (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Stained cells were analyzed with a FACS Caliber flow cytometer (Becton-Dickinson, San Jose, CA, USA) and data were analyzed using Flowjo software (Treestar, San Carlos, CA, USA).

2.7. Secretory protein analysis by antibody-based protein array

For the preparation of conditioned medium from normoxic and