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In vitro protection of adipose tissue-derived mesenchymal stem cells by erythropoietin

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ABSTRACT

Mobilization of stem cells and their differentiation into cardiomyocytes are known to have protective effects after myocardial infarction. The integrity of transplanted mesenchymal stem cells for cardiac regeneration is dependent on cell–cell or cell–matrix interaction, which is adversely affected by reactive oxygen species in an ischemic environment. Treatment with erythropoietin was shown to protect human adipose tissue derived mesenchymal stem cells in an ischemic injury in vitro model. The analyses indicated that expression of erythropoietin receptors played a pivotal role in erythropoietin mediated cell survival. In this study, the anti-apoptotic effect of erythropoietin on stem cells was analyzed in apoptosis-induced human mesenchymal stem cells. Apoptosis was induced in cultured adult human adipose tissue derived mesenchymal stem cells by hydrogen peroxide. A group of cultured cells was also treated with recombinant human erythropoietin in a concentration of 50 ng mL^{-1}. The degree of apoptosis was analyzed by flow-cytometry and immunohistochemical staining for Caspase 3. The average percentages of apoptotic cells were significantly higher in H_2O_2-induced stem cells than in cells co-cultured with erythropoietin ($63.03 \pm 4.96\%$ vs $29 \pm 3.41\%$, $p < 0.01$). We conclude that preconditioning with erythropoietin suppresses apoptosis of mesenchymal stem cells and enhances their survival.

Introduction

Myocardial infarction is a leading cause of morbidity and mortality worldwide. Attempts to repair infarcted myocardium using stem cells have been reported (Leri et al., 2005; Liao et al., 2007; Pasha et al., 2008; Jing et al., 2008; Robey et al., 2008). Mesenchymal stem cells (MSCs) represent a suitable sub-cell type for regeneration of infarcted myocardium. MSCs are self-renewing and clonal precursors of non-hematopoietic tissues (Uemura et al., 2006; Liao et al., 2007; Pasha et al., 2008; Hwangbo et al., 2010; Madonna and De Caterina, 2010). The promising therapeutic effect(s) of MSCs depend on their capacity to survive and to be incorporated in the target tissue. Cardiac tissue regeneration is carried out either by differentiation of transplanted cells toward cardiomyocytes or by their paracrine activity, which has the potential to replace myocardial mass, to form a functional vascular network, and to correct the ventricular geometry (Mazo et al., 2012). Although a large number of MSCs have been transplanted into infarcted hearts, it has been observed that only a limited number of cells MSCs survived for one week after injection. This situation has partly been attributed to poor viability and increased apoptosis of the transplanted MSCs in the ischemic environment (Rangappa et al., 2003; Leri et al., 2005; Xie et al., 2006; Pasha et al., 2008). Therefore, to develop strategies to improve the ability of MSCs to survive in this environment it is important for their proliferation and differentiation into cardiac phenotypes, which can lead to cardiac regeneration in the infarcted myocardium.

Previous studies have shown that systemic administration of erythropoietin (EPO) improves survival and cardiac function in the experimental myocardial infarction models. In addition to the

Abbreviations: dNTP, deoxynucleotide triphosphate; EDTA, ethylenediaminetetraacetic acid; EPO, erythropoietin; EPOR, erythropoietin receptor; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; H_2O_2, hydrogen peroxide; hAT-MSCs, human adipose tissue derived mesenchymal stem cells; HBSS, Hanks' balanced salt solution; HIF, hypoxia inducible factors; IBMX, isobutyl-methylxanthine; L-DMEM, low glucose Dulbecco's modified Eagle's medium; MEM, minimal essential medium; M-MuLV RT, Moloney Murine Leukemia Virus reverse transcriptase; MSC, mesenchymal stem cell; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ORO, oil red O; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI, propidium iodide; RTA, relative telomerase activity; RT-PCR, reverse transcription-polymerase chain reaction; TMP, thymidine monophosphate; TRAP, telomeric repeat amplification protocol.

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stimulation of erythropoiesis by EPO it has been shown that EPO has anti-apoptotic, anti-oxidative, and anti-inflammatory properties in various organs. Also, EPO enhances new vessel formation in cardiac ischemic injury (Moon et al., 2003; Sterin-Borda et al., 2003; van der Meer et al., 2004). EPO inhibits apoptosis and limits infarct size during ischemia and reperfusion through activation of various intracellular signaling pathways (Parsa et al., 2003). EPO enhances cardiac contractility and relaxation independently of the change in hematocrit values (Parsa et al., 2003). Inhibition of apoptosis in ischemic/hypoxic tissue is the major cytoprotective effect of EPO. Similar antiapoptotic and cardioprotective effects of EPO, independent of the hematopoietic effects, have been shown with carbamylated EPO, a non-erythropoietic derivative of EPO, and with helix B-surface peptide, a peptide mimicking the three dimensional structure of EPO (Fiordaliso et al., 2005; Ueba et al., 2010). Consequently, EPO may play an important role in enhancing survival of MSCs in an ischemic environment because of its prominent anti-apoptotic and cytoprotective effects. Because human adipose tissue derived mesenchymal stem cells (hAT-MSCs) abundantly express erythropoietin receptor (EPOR), preconditioning with EPO may have a role in extending survival of hAT-MSCs. In this study, we investigated whether EPO decreases apoptosis induced by hydrogen peroxide (H_2O_2) in cultured adult hAT-MSCs. H_2O_2 causes cellular changes similar to ischemia or anoxia in various cell types, and is used in stem cells as an oxidant or stimulant of apoptosis (Fandrey et al., 1994; Kim et al., 2006; Jiang et al., 2009a, 2009b; Peng et al., 2009; Karaoz et al., 2010).

Materials and methods

Isolation of hAT-MSCs

Human adipose tissues were obtained from subcutaneous tissue samples excised from standard surgical abdominal incision in eight healthy mothers undergoing uncomplicated elective cesarean delivery, and the procedures were approved by the Ethics Committee, University of Kocaeli (KOU-IAEK:13/22). To remove blood and residues, tissue samples were washed several times with Hanks' balanced salt solution (HBSS) (Invitrogen/Gibco, Paisley, UK) containing 5% penicillin/streptomycin (Invitrogen/Gibco, Paisley, UK) solution without calcium and magnesium ions. Tissue samples were cut into small blocks about 10 mm^3 in size for mechanical separations. The washed adipose tissues were finely minced in a 100-mm dish, and collected in a 50-mL conical tube for chemical decomposition. Enzymatic digestion was performed in HBSS supplemented with 5 mL of 0.075% collagenase type I (Invitrogen/Gibco, Grand Island, NY, USA) for 60 min in a shaking water bath at 37 °C. The digests were pipetted vigorously at 20 min intervals, and dissociation was monitored microscopically. After approximately 60 min, the cell suspensions were filtered through a 70 μm-cell strainer to separate single cells from debris and tissue fragments of undigested adipose tissue. Cells were resuspended in 10 mL of modified Eagle's medium (Invitrogen/Gibco, Paisley, UK) supplemented with 1% penicillin/streptomycin and 15% fetal bovine serum (FBS) (Invitrogen/Gibco, Paisley, UK), and were centrifuged for 8–10 min at 300 × *g*. Adipose cells were washed three times in minimal essential medium (MEM) (Invitrogen/Gibco, Paisley, UK). Viability of the adipose cells was determined using the trypan blue dye exclusion method and a hemocytometer. The cells isolated from eight samples were plated in separate 25 cm^2 culture flasks containing MEM with 100 U mL^{-1} penicillin (Invitrogen/Gibco, Paisley, UK), 0.1 mg mL^{-1} streptomycin (Invitrogen/Gibco, Paisley, UK) and 15% FBS. Three days after the initiation of culture, the medium was replaced with fresh medium, and subsequently replaced twice a week. When 80–90% confluence was reached in the primary cell culture, the cells were treated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Invitrogen/Gibco, Paisley, UK) for 3 min. The separated cells were collected, centrifuged and re-plated at the rate of 1:3–1:4 for subculture. After each passage, cells were counted. Viability and proliferation index of the cells were determined using a conventional hemocytometric method and a MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell growth assay (Karaoz et al., 2010).

Immunophenotype identification with flow cytometry

Undifferentiated MSCs were subjected to flow cytometry analysis to confirm that the hAT-MSCs maintain their immunophenotypic characteristics after growth in the culture. After the 3rd passage, stem cells were harvested and resuspended in their own culture medium at a concentration of 1×10^6 cells mL^{-1}. Flow cytometry was performed using a FACS Calibur Flow Cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed using CellQuest software (Becton Dickinson, San Jose, CA, USA). Debris and dead cells were gated out by forward- and side-scatter profiles. Immunophenotyping of the hAT-MSCs was performed with antibodies against the following human antigens: CD3, CD4, CD5, CD7, CD8, CD10, CD11b, CD13, CD14, CD15, CD19, CD29, CD33, CD44, CD45, CD71, CD73, CD90, CD106, CD123, CD133, CD138, CD166, and HLA-DR, HLA-A, HLA-B, HLA-C, HLA-G (Becton Dickinson, San Jose, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR)

Expression of mRNA of EPOR on MSCs was shown with RT-PCR. Total RNA was isolated from 3×10^6 hAT-MSCs at the 3rd passage by the high Pure RNA isolation kit (Roche, Mannheim, Germany). After isolation, total RNA concentrations were measured at 260 nm by a NanoDrop spectrophotometer (NanoDrop Technology, Wilmington, DE, USA). One microgram of total RNA was reverse-transcribed into cDNA using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche, Mannheim, Germany) with both oligo (dT) and random hexamers. The cDNA synthesis reaction was carried out immediately after RNA isolation, and the reaction mix consisted of 5 μg of RNA, 1 mM of deoxynucleotide triphosphates (dNTPs), 0.2 μg of primers, 20 units of riboblock inhibitor, and 200 units of Moloney Murine Leukemia Virus reverse transcriptase (M-MuLV RT). The RNA, primers, and dNTP mixture was denatured for 1 min at 90 °C. The temperature was lowered to 42 °C before the addition of M-MuLV RT enzyme and the riboblock inhibitor. The reaction was run for 1 h at 42 °C. For the second-strand synthesis, polymerase chain reaction (PCR) was performed with a PCR enzyme mix. Primers used for RT-PCR were as follows: EPOR forward 5′-GCTGTATCATGGACCACCTC-3′ and reverse 5′-GGTAGGAGAAGCTGTAGTTG-3′. The 25 μL PCR reaction mixtures consisted of 1×PCR buffer, 0.2 mM dNTP, 0.5 μM of each primer, 1.25 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Fermentase, Vilnius, Lithuania) and 2 μL of cDNA from the first strand reaction. An initial 5 min denaturation step at 94 °C was followed by cDNA amplification cycles including denaturation at 94 °C, annealing at an appropriate temperature, and elongation at 72 °C. The PCR reactions ended with a final elongation step at 72 °C. The PCR products were analyzed by agarose gel electrophoresis.

Cell proliferation and viability-MTT test

The proliferation rate of hAT-MSCs was determined by MTT test. MSCs from the 3rd passage were analyzed using an MTT Cell Growth Kit (Chemicon, Temecula, CA, USA). MSC suspensions