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Mesenchymal stem cells rescue injured endothelial cells in an in vitro ischemia–reperfusion model via tunneling nanotube like structure-mediated mitochondrial transfer

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ABSTRACT

Mesenchymal stem cells can be used as a novel treatment of ischemic vascular disease; however, their therapeutic effect and mechanism of action require further evaluation. Mitochondrial dysfunction has core functions in ischemia–reperfusion injury of the microvascular network. A recent discovery has shown that intercellular communication using tunneling nanotubes can transfer mitochondria between adjacent cells. This study aimed to investigate the tunneling nanotube mechanisms that might be involved in stem cell-mediated mitochondrial rescue of injured vascular endothelial cells. Using laser scanning confocal microscopy, mitochondrial transfer via a tunneling nanotube-like structure was detected between mesenchymal stem cells and human umbilical vein endothelial cells. Oxygen glucose deprivation and reoxygenation were performed on human umbilical vein endothelial cells, which induced mitochondrial transfer through tunneling nanotube-like structures to become frequent and almost unidirectional from mesenchymal stem cells to injured endothelial cells, thereby resulting in the rescue of aerobic respiration and protection of endothelial cells from apoptosis. We found that the formation of tunneling nanotube-like structures might represent a defense and rescue mechanism through phosphatidylserines exposed on the surface of apoptotic endothelial cells and stem cell recognition. Our data provided evidence that stem cells can rescue damaged vascular endothelial cells through a mechanism that has not yet been identified.

Introduction

Mesenchymal stem cell (MSC) can facilitate collateral reconstruction and angiogenesis in areas of ischemia; thus, MSC transplantation offers new prospects for ischemic vascular disease therapy (Lin et al., 2011; Liu et al., 2008; Nakano-Doi et al., 2010). Whether MSCs transdifferentiate into and replace lost vascular cells or perform a paracrine function by secreting proangiogenic factors is unclear (Dar et al., 2012; Kinnaird et al., 2004; Spees et al., 2003). The mechanism of action of MSCs, particularly in the interaction between vascular endothelial cells and grafted MSCs should be further investigated.

Among the many complex mechanisms underlying vascular endothelium ischemia–reperfusion injury, mitochondrial damage appears to contribute significantly to these pathological processes (Chan, 2005; Li et al., 2012; Zhang et al., 2008). Mitochondria are essential organelles that play prominent roles in biological processes such as aerobic metabolism, oxidative phosphorylation, and cell death pathways (Dyall et al., 2004; Galluzzi et al., 2012; Spees et al., 2006; Wen et al., 2013). Endothelial mitochondria have been recognized as playing critical roles in the signaling cellular responses to environmental cues, which may determine the endothelial function and fate, thereby influencing angiogenesis in ischemia–reperfusion injury (Kluge et al., 2013). Limited experimental data are available on the effects of stem cells on injured mitochondria in endothelial cells. Recent studies have discovered highly sensitive nanotubular structures named tunneling nanotubes (TNTs) that bridge adjacent animal cells, enabling them to form complex networks (Rustom et al., 2004). As a novel mechanism of cell–cell communication, TNTs facilitate the exchange of cellular components and signaling molecules between connected cells such as plasma membrane components, calcium ions, pathogens, and organelles, including mitochondria (He et al., 2011; Onfelt et al., 2006; Pasquier et al., 2013; Rustom et al., 2004; Vallabhaneni et al., 2012; Wang and Gerdes, 2012; Wang et al., 2010; Yasuda et al., 2010). We hypothesized that stem cells and post-ischemic endothelial cells

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interact with each other through the formation of TNTs and that this novel mechanism might be responsible for the beneficial effects exerted by engrafted stem cells.

In this work, we determined whether MSCs could repair post-ischemic endothelial cells with dysfunctional mitochondria by transferring functional mitochondria from healthy cells via TNT-like structures. Oxygen glucose deprivation (OGD) and reoxygenation (RO) were performed on human umbilical vein endothelial cell (HUVEC) cultures. MSCs were then co-cultured with injured the HUVECs. TNT-like structure-mediated mitochondrial transfer was observed, and the protective effects on the injured endothelial cells were further demonstrated. The beneficial effects of stem cell grafting on the treatment of ischemic vascular diseases might also be based on the TNT-like structure-mediated mitochondrial transfer rescue of damaged endothelial cells and not just through a paracrine or transdifferentiation mechanism. Our findings challenge the classical view of stem cell transplantation therapy for ischemic vascular diseases and might have implications in the treatment of other diseases with mitochondrial dysfunction.

Methods

Cell culture

The use of human tissue in this study was approved by the Research Ethics Committee of the Qilu Hospital of Shandong University. The human MSCs were isolated and cultured as previously described (Liu et al., 2008; Soleimani and Nadri, 2009). After obtaining written consent from each volunteer, the human MSCs were isolated from the bone marrow of the subjects using a density gradient (1.073 g/mL). The isolated cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM; Gibco, Scotland, UK) containing 10% fetal bovine serum (FBS; Hyclone, USA), and then incubated at 37 °C with 5% CO_2. The non-adherent cells were carefully removed after 48 h.

To induce mitochondrial dysfunction by mitochondrial DNA (mtDNA) mutation and depletion, MSCs were treated with 100 ng/mL ethidium bromide, 110 mg/mL sodium pyruvate, and 50 mg/mL uridine as previously described (Vallabhaneni et al., 2012). The efficacy of mitochondrial dysfunction was confirmed by cell death in a medium lacking pyruvate/uridine. The mtDNA-depleted MSCs were used for cocultures.

The HUVECs were obtained from the Key Laboratory of Cardiovascular Proteomics of Shandong Province (Cui et al., 2012). The cells were cultured in DMEM with 10% FBS, and incubated at 37 °C with 5% CO_2. The HUVECs were passaged every five days, and passages 4 to 8 were used for the subsequent experiments.

Identification of stem cells

The MSCs were analyzed by fluorescence-activated cell sorting (FACS) to evaluate the cell surface markers, as previously described. The MSCs were incubated with FITC-conjugated CD34 polyclonal antibody, PE-conjugated CD29 polyclonal antibody, PE-conjugated CD45 polyclonal antibody, PE-conjugated anti-human CD105 polyclonal antibody, and PE/Cy5-conjugated CD44 polyclonal antibody (BioLegend, San Diego, CA, USA). The MSCs expressed the antigens CD105, CD29, and CD44; however, they were negative for CD45 and CD34, in agreement with our previous study (Liu et al., 2008).

Cell label and lentiviral transduction

The HUVECs and MSCs were labeled for distinction before the co-cultivation. To investigate the protective effect of the MSCs on the injured HUVECs, the MSCs were incubated with lentiviral vector pWPT-enhanced green fluorescence protein (EGFP) (donated by the Institute of Molecular Medicine and Genetics, Shandong University School of Medicine, China) at a multiplicity of infection of 25, resulting in 96.1% ± 1.7% positive MSCs after 4 days of infection (Zou et al., 2009). The transduced cells were further sorted and purified in a FACSVantage flow cytometer (Becton Dickinson, USA) and then expanded.

To investigate the mitochondrial transfer, the MSC nuclei were stained with Hoechst 33342 (0.5 μg/mL, Sigma, San Francisco, CA, USA) at 37 °C for 30 min before co-cultivation. The mitochondria of the MSCs and the HUVECs were labeled. The pDsRed2-Mito vector (Clontech, Mountain View, CA, USA), which encodes a mitochondrial targeting sequence from subunit VIII of the cytochrome c oxidase (Rizzuto et al., 1995) (Mito) and a fusion of *Discosoma* sp. red fluorescent protein (DsRed2), was used to label the mitochondria of the MSCs. The pAcGFP1-Mito vector (Clontech, Mountain View, CA USA) encoding Mito and *Aequorea coerulescens* (AcGFP1) was used to label the mitochondria of the HUVECs. The two types of vectors were packaged into mature lentivirus by 293FT cells (Invitrogen, Carlsbad, CA, USA) and used to infect the HUVECs and MSCs, respectively. The transduced cells were purified by FACS, and then expanded.

In vitro ischemia–reperfusion model and co-culture model

In vitro ischemia–reperfusion was simulated by conducting OGD and RO on the HUVECs. Based on the preliminary experiments, the labeled HUVECs were placed at a density of 10,000 cells/mL in the culture medium in an anoxia chamber (1% O_2–94% N_2–5% CO_2, Thermo Forma, Marietta, OH, USA). The media were exchanged with D-Hanks and bubbled for 10 min with the identical gas mixture (1% O_2–94% N_2–5% CO_2). After 150 min of the OGD, the RO was performed by reinstating the cells under the normoxic conditions and the pre-OGD medium (Cselenyak et al., 2010). At 4 h of RO, an equal number of MSCs (10,000 cells/mL) was directly added to the damaged HUVECs. The co-cultures were treated with or without latrunculin-A (LatA) (10 nM, Sigma, USA) (Konishi et al., 2009; Lou et al., 2012) or Annexin V (10 μg/mL, SINO Biological, Beijing, China) (Yasuda et al., 2011) for 4 h. The medium containing LatA or Annexin V was changed, and the co-culture continued for 44 h. The HUVECs that did not receive MSCs after the OGD/RO were used as the controls.

Flow cytometry and sorting

To investigate the protective effect on the injured HUVECs, at least 2×10^6 co-cultured cells were analyzed using FACS analysis after co-culture for 48 h. The rescued HUVECs were sorted and purified based on their distinguished morphology from the MSCs and the EGFP-negative label (additional file 1: Figs. S1A–C). The sorted cells were cultured into chamber slides and visualized under a laser scanning confocal microscope to confirm that these cells had uniform short fusiform morphologies and were negative for EGFP (additional file 1: Fig. S1D). Freshly sorted HUVECs were used for subsequent cell apoptosis, viability, mitochondrial function, and genotype assays.

To investigate the mitochondrial transfer further, the exchange rate of mitochondria between the HUVECs and the MSCs was analyzed after co-culture for 48 h using FACS analysis. We could also distinguish the two different cells by their Hoechst 33342 label and different morphologies using FACS. To detect the Hoechst 33342 labeling cells through FACS analysis, we used the argon–ion laser to excite the fluorophore at 350 nm and to emit at 461 nm. The AcGFP1-expressing cells were excited at 475 nm and emitted at 505 nm, whereas the DsRed2-expressing cells were excited at 558 nm and emitted at 583 nm.

Cell apoptosis assay

After co-culture for 48 h, the cell apoptosis was analyzed using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen™, San Diego, CA, USA). The rescued HUVECs were resuspended in 1× annexin-binding buffer according to a previously published protocol (Zhou et al., 2012). Approximately 1 μL of PI and 5 μL of Annexin V