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Autophagy induction is a survival response against oxidative stress in bone marrow–derived mesenchymal stromal cells

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Abstract

Background aims. Bone marrow–derived mesenchymal stromal cells (BMSCs) are being extensively investigated as cellular therapeutics for many diseases, including cardiovascular diseases. Although preclinical studies indicated that BMSC transplantation into infarcted hearts improved heart function, there are problems to be resolved, such as the low survival rate of BMSCs during the transplantation process and in the ischemic region with extreme oxidative stress. Autophagy plays pivotal roles in maintaining cellular homeostasis and defending against environmental stresses. However, the precise roles of autophagy in BMSCs under oxidative stress remain largely uncharacterized. *Methods.* BMSCs were treated with H_2O_2, and autophagic flux was examined by means of microtubule-associated protein 1A/1B-light chain 3 II/I ratio (LC3 II/I), autophagosome formation and p62 expression. Cytotoxicity and cell death assays were performed after co-treatment of BMSCs by autophagy inhibitor (3-methyladenine) or autophagy activator (rapamycin) together with H_2O_2. *Results.* We show that short exposure (1 h) of BMSCs to H_2O_2 dramatically elevates autophagic flux (2- to 4-fold), whereas 6-h prolonged oxidative treatment reduces autophagy but enhances caspase-3 and caspase-6–associated apoptosis. Furthermore, we show that pre- and co-treatment with rapamycin ameliorates H_2O_2-induced caspase-3 and caspase-6 activation and cell toxicity but that 3-methyladenine exacerbates H_2O_2-induced cell apoptotic cell death. *Conclusions.* Our results demonstrate that autophagy is critical for the survival of BMSCs under oxidative conditions. Importantly, we also suggest that the early induction of autophagic flux is possibly a self-defensive mechanism common in oxidant-tolerant cells.

Key Words: *apoptosis, autophagy, bone marrow–derived mesenchymal stromal cell, oxidative stress, therapeutic strategy*

Introduction

Bone marrow–derived mesenchymal stromal cells (BMSCs) represent a self-renewing population of adult stem cells that are capable of differentiating into cells of the mesodermal lineages, such as osteocytes, chondrocytes and adipocytes, as well as cells of other non-mesodermal lineages (1–3). Emerging evidence has demonstrated the potential application of BMSC transplantation from bone marrow to injured tissues, such as the infarcted heart, as an effective treatment (4,5). Although BMSCs display promising therapeutic effects, their poor survival capacity during and after transplantation is an important issue of concern and deserves further investigation. The stress conditions, such as hypoxia, serum deprivation and oxidative stress during isolation and processing, cause the low survival rate of BMSCs (4,6,7). Furthermore, the microenvironment of the injured myocardium with high oxidation level in patients promotes the apoptosis of the transplanted cells as well. As reported, 6×10^7 BMSCs were transplanted into infarcted porcine hearts, but only a limited number of cells survived after 7 days, and myocardial function improvement was marginal (8). Consequently, approaches to increase the survival of transplanted BMSCs to withstand the harshness of the stresses are extremely important (8,9).

Autophagy, a lysosome-dependent degradation pathway, is an evolutionarily conserved mechanism and essential cellular homeostatic process that performs continuous recycling of protein aggregates, lipids and dysfunctional organelles (10). In mammalian cells, there are three major autophagic pathways, which are interconnected each other: macroautophagy, microautophagy and chaperone-mediated autophagy. Macroautophagy (referred to hereafter as autophagy), the most widely studied and

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characterized, involves the sequestration of cytoplasmic cargo (proteins or organelles) into double-membrane–bounded vacuoles, termed the autophagosomes. The autophagosomes subsequently fuse with lysosomes to form autolysosomes, in which the contents are hydrolytically degraded by lysosomal enzymes. Accumulating evidence has demonstrated that autophagy is a critical homeostatic response to maintain general cellular processes under stressed conditions. Furthermore, the strong associations between autophagy defectiveness and pathological conditions, such as aging, cancer, neurodegenerative diseases and cardiac disorders, have also been proved by previous studies (11,12).

Recent studies have indicated that autophagy also plays pivotal roles in stem cell maintenance and differentiation. Adult stem cells are maintained in a quiescent state and have a long life span, but they have inadequate capability to remove cellular aggregates and wastes, which strongly indicates that autophagy is more needed in stem cells than in other cell types (13,14). Some recent reports indicated that hypoxia and serum deprivation induce autophagy, which promotes stem cell survival (15,16). However, the underlying mechanism of how autophagy is induced in BMSCs under stressed situations is still not clear. Moreover, little is known about the precise role of autophagy in BMSCs under oxidative conditions. In the present study, we show for the first time that oxidative stress rapidly enhanced autophagy at an early stage but that prolonged oxidative exposure reduced autophagy and increased apoptosis at a late stage. Furthermore, we also suggested that induction of autophagy may play important roles in protecting cells against oxidative stress in oxidant-tolerant cells.

Methods

Materials

H_2O_2, 3-MA, rapamycin, bafilomycin A1 and MTT were obtained from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium (low glucose), trypsin–ethylenediaminetetra-acetic acid solution (0.25%), penicillin/streptomycin and fetal bovine serum were purchased from Hyclone. Opti-MEM reduced serum medium, Lipofectamine RNAiMAX reagent, Lipofectamine LTX reagent and TRIzol reagent were purchased from Life Technologies (Grand Island, NY, USA). The Bradford protein assay kit was purchased from BOSTER (Wuhan, China). The primary antibodies used in this study were anti-LC3 (rabbit polyclonal; Abcam, Cambridge, MA, USA), anti-*ATG9A* (rabbit polyclonal, Abcam), anti–β-actin (mouse monoclonal, BOSTER), anti-*ATG7* (rabbit polyclonal, Abcam), Beclin-1 (mouse monoclonal; BD Bioscience, San Jose, CA, USA), anti-p62 (rabbit polyclonal, Abcam) and caspase-3 (rabbit polyclonal; Santa Cruz, Santa Cruz, CA, USA; 1:1000). Horseradish peroxidase–conjugated secondary antibodies (BOSTER) and an ECL chemiluminescence kit (Applygen Technologies Inc, Beijing, China) were used.

BMSC preparation and treatment paradigm

As previously described (17), we isolated BMSCs from the bone marrow of the femurs and tibias of C57BL/6 mice (8–10 weeks old; Beijing HFK Bioscience Co, Ltd, Beijing, China) after euthanization and expanded the cells for use between passages 3–6. Briefly, epiphyses were removed and bony shafts were flushed repeatedly with the use of complete media. Cells were then strained through a 30-μm nylon mesh, collected and spun at 300 rpm for 5 min. The cell pellet was resuspended in fresh cell growth medium (low-glucose Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% penicillin/streptomycin) and maintained in a humidified CO_2 incubator (5% CO_2, 37°C). After 24 h, complete media were replaced and changed every 3–4 days after. At 80–90% confluence, BMSCs were trypsinized and further expanded at a ratio of 1:2.

BMSCs during three to six expansion passages were exposed to 100 or 500 μmol/L of H_2O_2 in the presence or absence of 50 nmol/L of rapamycin (autophagy activator) or 5 mmol/L of 3-MA (autophagy inhibitor) for the duration of the experiment. Untreated or vehicle-treated cells were used as controls.

Western blot analysis

BMSCs were seeded in six-well plates, and after various treatments they were lysed with the use of radioimmunoprecipitation assay buffer containing protease inhibitor. Cell lysates containing equal amounts of proteins were loaded into each lane and separated on a 4–20% gradient sodium dodecyl sulfate–polyacrylamide electrophoresis gel. After separation, proteins were transferred to nitrocellulose membrane, and nonspecific binding sites were blocked by treatment with 5% nonfat dry milk. The membranes were then incubated with primary antibodies directed against LC3 (rabbit polyclonal, 1:1000 dilution), p62 (rabbit polyclonal, 1:2000 dilution) or β-actin (mouse monoclonal, 1:5000 dilution). The primary antibody treatments were followed by treatment with a horseradish peroxidase–conjugated secondary antibody for 1 h at room temperature. The membranes were incubated with a Western blot detection system and exposed to single-emulsion film (Biomax MR; Sigma). Band