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Autophagy mediates enhancement of proangiogenic activity by hypoxia in mesenchymal stromal/stem cells

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ABSTRACT

Mesenchymal stromal/stem cells (MSCs) have been promising source for regenerative cell therapy in ischemic diseases. To improve efficacy of MSC therapy, various priming methods have been developed, and hypoxic priming has been reported to enhance therapeutic efficacy of MSCs by increasing secretion level of growth factors and cytokines. Recently, it has been reported that bone marrow MSCs primed with hypoxic condition show an increase of autophagy. Here, we addressed whether proangiogenic activity increased by hypoxic condition is associated with autophagy. Wharton's jelly derived MSCs primed with hypoxia showed increase of autophagy with increased hypoxia inducible factor-1α level, and conditioned medium (CM) derived from these cells showed increased levels of migration and tube formation of human umbilical vein endothelial cells (HUVECs) compared to non-primed MSCs-derived CM. Pre-treatment with autophagy inhibitor 3-methyladenine or chloroquine prior to exposure of hypoxia resulted in reduction of migration and tube formation of HUVECs. CM obtained under hypoxic condition from MSCs in which autophagy activity was inhibited by ATG5 and ATG7 siRNA treatment also showed decrease of migration and tube formation of HUVECs. Accordingly, secretion levels of angiogenin and VEGF that were markedly increased upon hypoxia exposure was decreased by ATG5/7 knockdown. Therefore, it may be suggested that autophagy plays an important role in hypoxia-driven enhancement of paracrine effect of MSCs.

1. Introduction

Mesenchymal stromal/stem cells (MSCs) have been widely studied as a promising source for cell therapy in disorders that are related to insufficient angiogenesis such as stroke, chronic wounds and myocardial infarction [1]. MSCs derived from various tissue sources have been shown to exert proangiogenic therapeutic efficacy in ischemic disease in preclinical and clinical settings [2–6]. A predominant aspect of therapeutic mechanisms of MSCs has been frequently associated with their paracrine secretion capacity [1,2]. Direct implantation of MSCs has been used as a treatment strategy in angiogenesis related disease models [2,6,7], but several studies have also reported that MSC-conditioned medium without cell implantation can provide another treatment option for improvement of tissue repair [8,9]. In order to improve therapeutic effect of MSCs, various methods have been developed for priming or modifying MSCs before cell therapy [10,11]. Priming MSCs under hypoxic condition has been reported to enhance secretion of growth factors and cytokines [12], survival [13], and therapeutic effect in ischemic disease animal model [14]. Recently, it has been reported that hypoxia treatment of bone marrow MSCs induces autophagy in HIF-1α-dependent manner, with increased cell survival [15]. Autophagy is a self-cannibalization process that allows orderly degradation and recycling of dysfunctional cellular organelles or macromolecules to maintain cellular homeostasis [16,17]. Recently, autophagy has been reported to mediate osteogenic [18] or adipogenic [19] differentiation of MSCs. However, it has not been studied whether enhanced autophagy in MSCs primed with hypoxic condition is correlated with enhanced proangiogenic activity. In this study, we investigated whether hypoxic priming of MSCs increases paracrine potency through autophagy enhancement, and identified which proangiogenic factors play key roles in this process.

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2. Materials and methods

2.1. Cell culture

The isolation and culture of Wharton's jelly MSCs (WJ-MSCs) were performed as described previously [5]. WJ-MSCs were cultured in DMEM (Gibco BRL, Grand Island, NY) with 20% FBS (Merck, Darmstadt, Germany) and 100 units of penicillin-streptomycin (Gibco BRL) at 37 °C under 5% CO_2 in a humidified atmosphere. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously [5]. HUVECs were cultured in M199 medium (Gibco BRL) supplemented with 20% FBS (Gibco BRL), 30 μg/ml endothelial cell growth supplements (BD Biosciences, San Jose, CA), and 90 μg/ml heparin (Sigma, St. Louis, MO).

2.2. Hypoxic priming and treatment of autophagy inhibitors

WJ-MSCs were seeded at 7.8×10^5 cells in 60 mm culture dish (36 cells/mm^2) with culture media. After 24 h, the cells were washed twice with PBS and medium was pretreated with 5 mM 3-methyladenine (3 MA; Sigma) or 10 μM chloroquine (CQ; Sigma) in DMEM containing 20% FBS for 48 h. Then, the treated cells were washed twice with PBS and medium was replaced with serum free EBM-2 and cultured further for 24 h in normoxic or hypoxic condition. To achieve hypoxic condition, the cells were incubated in a hypoxia chamber (Thermo electron, USA) with a gas mixture containing less than 1% O_2. To obtain conditioned medium (CM), the culture supernatant was collected, centrifuged at 3200 rpm for 15 min at 4 °C, and kept in −70 °C until use.

2.3. Western blot analysis

Cells were harvested by scraping and lysed in a buffer containing 50 mM Tris–HCl pH 8.0, 150 mM NaCl, 1% Triton X 100, 1 mM Na_3VO_4, 1% sodium deoxycholate, 0.1% SDS, 1 μg/ml pepstatin A, 50 mM NaF, 0.5 mM EDTA, 1 mM EGTA and Protease inhibitor cocktail (Roche, Basel, Switzerland). Equal amounts of protein were resolved on a SDS-PAGE gel and transferred to a nitrocellulose membrane. Then, the membranes were blocked by 5% skim milk for 1 h at 37 °C and then incubated with primary antibodies against hypoxia inducible factor-1α (HIF-1α; Cell Signaling Technology, Danvers, MA), LC3B (Cell Signaling Technology), ATG5 (Cell Signaling Technology), ATG7 (Cell Signaling Technology), and β-actin (Sigma) at 4 °C overnight. Then, the membranes were incubated with respective secondary antibodies (GeneTex, Irvine, CA, USA) for 2 h at 37 °C. The binding was detected by the enhanced chemiluminescence system (ECL Plus kit, GE Healthcare, Piscataway, NJ, USA).

2.4. Immunofluorescence for LC3B$^+$ punta analysis

Cells grown on cover slip were fixed with 4% (v/v) paraformaldehyde for 30 min, permeabilized with methanol at −20 °C for 10 min, and then covered with 1% BSA for 30 min at room temperature for blocking. Then, the cells were incubated with primary antibody against LC3B (Cell Signaling Technology) at 4 °C overnight, and then probed with Alexa Fluor 594 goat anti-rabbit secondary antibodies (Thermo scientific, Foster city, California) at room temperature for 2 h. Nuclei were stained with DAPI (Sigma). Fluorescent signals were detected using fluorescence microscopy (Olympus BX51, Middlesex, UK). The percentages of autophagic cells were calculated as the number of cells with LC3-positive punta (≥10 dots/cell) divided by the total number of cells in each 3 random fields.

2.5. Cell migration assay

Cell migration assay was performed in a modified Boyden chamber as described previously [20]. Each CM was placed in bottom chamber and detached serum starved HUVECs (2×10^4 cells) were placed in the upper chamber. Cell migration was allowed for 5 h, and the migrated cells were fixed and stained with Diff-Quik solution (Sysmex Co., Kobe, Japan). The migrated cells were photographed and counted in five randomly selected fields.

2.6. Tube formation assay

Prechilled Matrigel (BD Bioscience) was placed in a prechilled 96 well plate (50 μl) and incubated for 30 min. Detached serum starved HUVECs were suspended in each CM and added to the top of the solidified Matrigel. After 12 h, the formed tubes were photographed and images were analyzed using the Image J software (http://rsb.info.nih.gov/ij/) to determine the tube length.

2.7. RNA interference

Small interfering (si) RNAs targeting ATG5, ATG7, angiogenin, VEGF and control siRNAs (siCTL) were obtained from Bioneer (Daejun, Korea). Sequences of the double-stranded siRNAs were as follows: targeting for ATG5, sense 5′-GGA AUA UCC UGC AGA AGA A dTdT-3′ and antisense 5′-UUC UUC UGC AGG AUA UUC C dTdT-3`; for ATG7, sense 5′-GAA GAU AAC AAU UGG UGU AUU dTdT-3′ and antisense 5′-AAU ACA CCA AUU GUU AUC UUC dTdT-3`; for angiogenin, sense 5′-ACG UUG UUGUUG CUU GUG A dTdT-3′ and antisense 5′-U CAC AAG CAA CAA CAA CGU dTdT-3`; for VEGF, sense 5′-GGA GUA CCC UGA UGA GAU C dTdT-3′ and antisense 5′-G AUC UCA UCA GGG UAC UCC dTdT-3`. siCTL (sense 5′-GUU CAG CGU GUC CGG CGA G dTdT-3′ and antisense 5′-C UCG CCG GAC ACG CUG AAC dTdT-3′), which does not have any known homology to mammalian genes, was used as a negative control. Cells were seeded at a density of 36 cells/mm^2 and cultured for 24 h. Then, the cells were transfected with each siRNA (50–100 nM) using lipofectamine RNAiMAX Transfection Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After 4 h, the transfection medium was removed, and the cells were washed with PBS twice and then incubated in fresh medium. After 48 h, the cells were washed with PBS and added with fresh EBM2 and cultured further for 24 h in normoxic or hypoxic condition. CMs collected were used for in vitro angiogenesis assays or blotted on a nitrocellulose membrane using a 96-well Bio-dot apparatus (Bio-Rad Laboratories). The membrane was blocked in 5% skim milk and incubated with specific primary antibodies, anti-angiogenin (Millipore, Billerica, MA) and anti-VEGF (R&D system, Minneapolis, MN), and then with horseradish peroxidase-conjugated secondary antibodies. Immunoreactive dots were visualized using an ECL kit (GE Healthcare).

2.8. Antibody-based protein array analysis

Antibody-based protein array was performed for CM using Human Angiogenesis Array Kit (ARY700, R&D system) according to the manufacturer's instructions.

2.9. Data and statistical analysis

All experiments were repeated in triplicate. Data were presented as mean ± SE of representative experiments. Differences between groups were analyzed by independent *t*-test or one-way ANOVA with Tukey test using GraphPad Prism 5 (GraphPad Software) for most experiments. P values less than 0.05 were

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