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Autophagy in endothelial progenitor cells is cytoprotective in hypoxic conditions

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Wang HJ, Zhang D, Tan YZ, Li T. Autophagy in endothelial progenitor cells is cytoprotective in hypoxic conditions. Am J Physiol Cell Physiol 304: C617–C626, 2013. First published December 26, 2012; doi:10.1152/ajpcell.00296.2012.—Endothelial progenitor cells (EPCs) may be incorporated into local vessels to enhance angiogenesis within ischemic tissue. Recently, EPC transplantation has become a potential therapy for improving tissue function in cardiovascular disease. However, the mechanisms of proliferation, differentiation, and survival of EPCs in a hypoxic microenvironment remain unclear. In this study, CD34+ VEGFR-2+ EPCs were isolated from mononuclear cells of human umbilical cord blood, and differentiation to endothelial cells was induced with VEGF. When EPC autophagy was inhibited with 3-methyladenine (3-MA) under normoxic conditions, proliferation and viability of the cells were decreased, and the cells failed to differentiate into endothelial cells. Under hypoxic conditions (1% O2), Beclin-1 expression of the cells was upregulated and both MDC-labeled and LC3-positive puncta and autophagic ultrastructures in the cells increased significantly. The number of lysosomes also increased in hypoxia-exposed cells. When autophagy was inhibited with 3-MA under hypoxic conditions, the number of apoptotic cells increased, and the number and size of lysosomes decreased. Conversely, apoptosis of the hypoxic EPCs was reduced when autophagy was induced by pretreatment with rapamycin. These results demonstrate that autophagy is involved in proliferation and differentiation of EPCs. Furthermore, hypoxia activates autophagy, promoting EPC survival by inhibiting apoptosis. Enhancing autophagy with hypoxia preconditioning may be beneficial for survival of the transplanted EPCs in a local hypoxic environment.

autophagy; endothelial progenitor cells; proliferation; differentiation; survival

CARDIOVASCULAR DISEASES with their high morbidity and mortality are a socioeconomic burden for modern nations. Cell-based therapy has emerged as a promising therapeutic tool for treatment of ischemic cardiovascular disease. In recent years, more and more attention in cardiovascular regenerative medicine has focused on endothelial progenitor cell (EPC) transplantation to promote beneficial angiogenesis. Postnatally, EPCs are involved in angiogenesis during body development, tumor growth, recovery from vascular trauma, and inflammatory and ischemic diseases. In response to stimulation of chemokines and some growth factors, EPCs are mobilized from the bone marrow into peripheral blood, then home to the local tissue where angiogenesis is occurring and incorporate into the endothelium of the existing vessels. Local growth factors induce EPCs to proliferate and differentiate into mature endothelial cells, promoting the formation of new vessels, establishment of collateral anastomosis, and repair of vessel trauma (45). However, the number of EPCs in some patients with cardiovascular disease is reduced and EPC function is attenuated as a consequence of exposure to risk factors (38). Recently, EPC transplantation has become a potential therapy for treatment of ischemic disease (20). Several studies show that EPC transplantation provides a modest benefit for treatment of myocardial infarction (19, 41), limb ischemia (21), and diabetic retinopathy (6), compensating for a shortage of endogenous EPCs. Angiogenic effects of the transplanted EPCs include differentiation into endothelial cells and production of cytokines that stimulate the existing endothelial cells to form new vessels through paracrine effects.

Several different approaches to cell-based therapies have been limited by poor survival of the engrafted cells (9). For example, at day 4 after engraftment into the left ventricle of mice, only 0.44% of infused human mesenchymal stem cells (MSCs) were found within the myocardium (43). Intra-arterially delivered rat MSCs decreased to 14% of their initial numbers at day 3 (44). In rat models of myocardial infarction, only 0.1% of bone marrow-derived stromal cells remained after intramyocardial injection for 10 wk and ~0.1% of cells were detected after intravenous infusion for 6 wk (7). At 4 wk after transplantation, MSCs were not present in scar tissue of infarcted rat hearts (2). In patients with myocardial infarction, only 1.3 to 2.6% of unfractionated bone marrow cells and 4%–39% of CD34+ cells remained after intracoronary transfer (16). Cumulatively, these animal and human studies suggest that benefits of cell therapy are limited by massive death of engrafted cells. To improve these outcomes, it is necessary to elucidate mechanisms of EPC survival after transplantation and to find new ways for improving survival of the implanted cells.

Autophagy is a cellular pathway involved in protein and organelle degradation, with connections to human physiology and disease (25). Autophagy may be divided into macroautophagy, microautophagy, and chaperone-mediated autophagy based on the pathways by which cargo is delivered to lysosomes. In macroautophagy, the autophagosome precursor encompasses a portion of the cytoplasm or organelles and then closes to form the autophagosome. The autophagosome fuses with the lysosome to form an autolysosome where the engulfed cargo and the inner membrane of the autophagosome are degraded by acid hydrolases (3). Autophagy occurs at basal, constitutive levels and is upregulated under physiological stress such as nutrient deprivation and growth factor withdrawal (26). Under these conditions, autophagy plays important roles in getting rid of the degenerated or senescent organelles and aggregation-prone proteins and supplying energy for cellular activities. Autophagy malfunction contributes to the pathogenesis of cardiovascular and neurodegenerative disorders (31). In ischemic cell injury, upregulation of autophagy delays both apoptosis and necrosis (22). Uncontrolled autophagy may...
lead to cell death, presumably due to excessive degradation of cellular constituents (31). Recently, in vitro experiments have demonstrated that autophagy of endothelial cells promotes angiogenesis (10). However, whether autophagy plays a cytoprotective role in the proliferation, differentiation, and survival of EPCs in a hypoxic environment is unclear.

This investigation was designed to examine changes in macroautophagy (hereinafter termed “autophagy”) of cord blood-derived EPCs during differentiation into endothelial cells and to evaluate the effects of autophagy on EPC survival after hypoxia treatment. Because the number of dying or dead cells increases significantly after long-term hypoxia, the results of experiments in determining the protective effect of autophagy on EPC survival may be difficult to discern. Therefore, cells were treated with short-term severe hypoxia (1% O₂, 2 h). Here we report that inhibition of autophagy reduces proliferation and differentiation of EPCs under normoxic conditions. Under hypoxic conditions, the level of autophagy of EPCs increases significantly, and enhancement of autophagy increases EPC survival.

MATERIALS AND METHODS

Isolation of mononuclear cells. The present study was approved by the Ethics Committee of Shanghai Health Hospital for Women and Children. Written informed consent was obtained from the parents of the newborns. Human umbilical cord blood samples were collected from normal full-term deliveries. Sodium heparin (10 U/ml) was added into blood for anticoagulation. After being allowed to settle for 30 min at room temperature, the upper part of blood was layered onto 1.076 Percoll solution (Amersham Pharmacia Biotech, Uppsala, Sweden). After centrifugation for 30 min at 250 g, the mononuclear cells were harvested. The cells were resuspended in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 50 ng/ml vascular endothelium growth factor (VEGF; R&D Systems), 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Then, the cells were seeded into gelatin-coated culture dishes at a density of 1 × 10⁵ cells/cm² and incubated in a 5% CO₂ humidified incubator at 37°C. Half of the medium was changed with the complete medium every 2 or 3 days.

Sorting of EPCs. After incubation for 7 days, the cells were harvested by digestion with 0.125% trypsin and 0.01% EDTA. The suspended cells were incubated with polyclonal rabbit anti-human vascular endothelial growth factor receptor-2 (VEGFR-2) antibody and mouse anti-human CD34 antibody (1:100; Diagnostica) or mouse anti-human CD133 antibody (1:100; Diagnostica) for 50 min at 4°C. After being washed, the cells were incubated with goat anti-rabbit IgG-PE and goat anti-mouse IgG-FITC (1:100; Chemicon) for 30 min at 4°C. Following centrifugation for 10 min at 250 g, the cells were resuspended with DMEM containing 2.5% FBS, CD34⁺−VEGFR-2⁺ and CD133⁺−VEGFR-2⁻ cells were analyzed by flow cytometry, and CD34⁺−VEGFR-2⁻ cells were collected by cell sorting (FACS Aria, BD Biosciences). Differentiation of EPCs into endothelial cells was determined by acquisition of CD31 expression using immunostaining after incubation for 2 wk.

Proliferation assay. Synchronization of the cells was performed with the method described by Olszewska et al. (27). When cells reached 80% confluence, they were incubated for 6 h at 4°C. The cells were collected and randomly divided into control and 3-methyladenine (3-MA) treatment groups. For the latter, 5 mM 3-MA (Sigma), an inhibitor of class III PI3K that prevents formation of autophagosomes, was added to the medium (30). Subsequently, cells were returned to normal growth conditions and incubated for an additional 12 h before harvest. After addition of precooled 95% ethanol, the cells were placed at 4°C overnight. The cells were mixed with 1 ml propidium iodide (PI; 1 mg/ml; Sigma, Deisenhofen, Germany) and then incubated at 4°C for 20 min. After centrifugation, the cells were resuspended at 2 × 10⁶ cells/ml. Cell cycle was analyzed immediately by flow cytometry.

Fig. 1. CD34⁺−VEGFR-2⁺ endothelial progenitor cells (EPCs) isolated from mononuclear cells of cord blood. A: percentages of CD34⁺−VEGFR-2⁺ cells and CD133⁺−VEGFR-2⁺ cells in cord blood-derived mononuclear cells are 0.83% and 0.62%, respectively. B: microphotographs of CD133⁺−VEGFR-2⁺ cells (top) and CD34⁺−VEGFR-2⁺ cells (bottom). C: after induction with 50 ng/ml VEGF for 10 days, the sorted CD34⁺−VEGFR-2⁺ EPCs seeded onto gelatin-coated culture dishes were grown to endothelium-like cells. Bars, 10 μm.
Expression of Beclin-1. Beclin-1, a phylogenetically conserved protein, is regarded as a specific autophagic gene. To assay expression of Beclin-1, cells were fixed with 4% paraformaldehyde for 10 min and then permeabilized with 0.25% Triton X-100 in PBS for 10 min. Cells were then washed and incubated with polyclonal antibody against Beclin-1 (1:200; Proteintech Group, Chicago, IL) overnight at 4°C. Subsequently, the cells were incubated with FITC-labeled IgG for 1 h and the nuclei were counterstained with DAPI (1:1,000; Sigma) at room temperature. Intensity of fluorescence-labeled Beclin-1 in the cells was examined with a confocal laser scanning microscope.

To examine Beclin-1 mRNA expression, total RNA from the hypoxia-treated cells was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Primers specific for the human Beclin-1 gene were used (human Beclin-1 primers forward, 5’-CAAGATCCTGGACCCTGTC-3’ and reverse, 5’-TGGCCTTTCTGTTGACATCA-3’). The first-strand cDNA was synthesized using T-primed first strand kit (Amersham Pharmacia Biotech) and then amplified by Taq DNA polymerase (Advantage cDNA Polymerase Mix, Invitrogen) in 20 μl reaction mixture containing 10 ml 2× Master Mix (Invitrogen), 5 pM Beclin-1 primers (or 6 pM GAPDH primers), and 1 μl (25 ng) of cDNA. Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using a PCR thermal cycler (MWG Biotech, Ebersberg, Germany). After a reverse transcription reaction at 70°C for 15 min and an initial denaturation at 95°C for 15 min, a 40-cycle PCR reaction was carried out using the following amplification profile: denaturation at 94°C for 45 s, annealing at 58°C for 45 s, extension at 72°C for 45 s. For GAPDH primers, a 35-cycle amplification was performed with annealing at 63°C. Then, the PCR products were electrophoresed on 1.5% agarose gel, stained with 0.5 μg/ml ethidium bromide, visualized under ultraviolet light, and photographed. Changes of Beclin-1 expression were determined with MxPro quantitative PCR software (version 4.0; Stratagene).

MDC staining. Autophagosomes in the cells were detected with monodansylcadaverine (MDC) staining using the method as previously described (5). Briefly, the cells were incubated with 0.05 mM MDC (Sigma) in Hanks’ buffered salt solution at 37°C for 10 min. After being washed three times with 0.1 M PBS, MDC-labeled autophagosomes were examined with a confocal laser scanning microscope. The cells containing MDC-labeled autophagosomes were counted in ×100 fields (five sequential fields were counted and averaged over coverslip) for three coverslips in each experiment as a percentage of total cell count.

LC3 staining. Microtubule-associated protein 1 light chain 3 (LC3) is expressed mainly on autophagosome and is used as a specific marker for autophagic structures (18). To assess LC3 localization, the cells were fixed in 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. Nonspecific binding sites were blocked with goat serum. The cells were fixed in 4% paraformaldehyde and then permeabilized with 0.5% Triton X-100. Subsequently, the cells were incubated with 0.05 mM monodansylcadaverine (MDC) staining using the method as previously described (5). Briefly, the cells were incubated with 0.05 mM MDC (Sigma) in Hanks’ buffered salt solution at 37°C for 10 min. After being washed three times with 0.1 M PBS, MDC-labeled autophagosomes were examined with a confocal laser scanning microscope. The cells containing MDC-labeled autophagosomes were counted in ×100 fields (five sequential fields were counted and averaged over coverslip) for three coverslips in each experiment as a percentage of total cell count.

Transmission electron microscopy. To prepare cells for analysis by transmission electron microscopy, a pellet of cells was prefixed with 2.5% glutaraldehyde in 0.1 M PBS overnight at 4°C and postfixed in 1.5% cacodylate buffer (pH 7.4) containing 1% osmium tetroxide and 5% potassium ferricyanide at 4°C for 1 h. Then the cells were washed three times with cacodylate buffer and then dehydrated in an ascending series of ethanol, followed by propylene oxide. The samples were finally embedded in epoxy resin (Taab, Reading, UK). Ultrathin sections were cut with an Ultracut E ultramicrotome (Reichert-Jung, Austria) and placed on 200-mesh copper grids. The sections were stained with 1% aqueous uranyl acetate and lead citrate and examined with a Jeol-1010 electron microscope (Jeol, Tokyo, Japan).

Table 1. Numbers of EPCs in the phases of the cell cycle

<table>
<thead>
<tr>
<th>Group</th>
<th>G0/G1 Phase, %</th>
<th>S Phase, %</th>
<th>G2/M Phase, %</th>
<th>Proliferation Index, %</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>75.94 ± 3.25</td>
<td>9.39 ± 1.29</td>
<td>14.67 ± 1.72</td>
<td>24.06 ± 3.25</td>
</tr>
<tr>
<td>3-MA</td>
<td>86.45 ± 3.71*</td>
<td>5.54 ± 0.63*</td>
<td>8.01 ± 0.92*</td>
<td>13.55 ± 3.71*</td>
</tr>
</tbody>
</table>

*Values are means ± SD; n = 3. The total percentage of cells in phases S and G2/M represents proliferation index. EPCs, endothelial progenitor cells; 3-MA, 3-methyladenine. *P < 0.05 vs. control group.
1% buffered osmium tetroxide. Then, the specimens were dehydrated through graded ethanol and embedded in epoxy resin. Ultrathin sections were prepared and stained with uranyl acetate and lead citrate. Autophagosome precursors, autophagosomes, and autolysosomes in the cells were examined with a transmission electron microscope (CM120, Philips, Eindhoven, Holland). Sectional areas of the autophagic structures were measured with a VLCDS image analyzer (Leica), and the ratios of the cross-sectional area of the autophagic structures to that of the cytoplasm were calculated. The autophagic structures were examined in 200 cells for each group.

**Labeling of lysosomes.** To evaluate the relation of lysosomes to the formation of autophagic structures, lysosomes in the cells were labeled with LysoSensor green (Invitrogen). After hypoxia treatment, the cells were incubated in 1 μM prewarmed LysoSensor green for 30 min. Hoechst 33342 (1:1,000; Invitrogen) was added to the dishes and the cells continued to be incubated for 20 min. The loading solution was then replaced with DMEM, and the distribution of the labeled lysosomes was examined using a confocal laser scanning microscope. Lysosomes were counted in 20 cells (five sequential ×100 fields were counted and averaged per culture dish) for three culture dishes.

**Analysis of apoptosis.** The cells were divided into control, hypoxia, and 3-MA groups. In the hypoxia group, cells were treated with hypoxia as above for 1 h. In the 3-MA group, cells were treated as above. The cells were incubated with 1 μg/ml FITC-Annexin V (BD Biosciences) and 1 μg/ml PI for 10 min. The apoptotic cells were analyzed by flow cytometry. For ethidium bromide and acridine orange (EB/AO) staining, a suspension of the cells was dropped onto slides, and a mixture of EB and AO solutions was then added. The

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**Fig. 3.** Beclin-1 expression of EPCs after hypoxic treatment. A: Beclin-1 immunostaining. The expression of Beclin-1 in the hypoxia groups increased. After pretreatment with 3-MA, Beclin-1 expression of the hypoxic cells was weaker. Bar, 10 μm. B: RT-PCR analysis of Beclin-1 mRNA expression. The levels of Beclin-1 mRNA expression increased in the hypoxia groups. After treatment with 3-MA, Beclin-1 mRNA expression in the hypoxic cells decreased. C: graph represents statistical results of RT-PCR analysis of Beclin-1 mRNA expression. In 3-MA-treated group, cells were incubated with 5 mM 3-MA for 1 h and then treated under hypoxia for 1 h. The experiment was repeated three times. *P < 0.05 and **P < 0.01 vs. control group; †P < 0.05 vs. hypoxia 30 min group; #P < 0.05 and ##P < 0.01 vs. hypoxia 1 h group.
stained cells were immediately examined using a fluorescent microscope. The percentages of viable, early apoptotic, late apoptotic, and necrotic cells were calculated respectively by the following formula: percentage (%) = number of viable, early apoptotic, late apoptotic, or necrotic cells/number of all counted cells (32). The experiment was repeated three times.

In another experiment, the cells were divided into control, hypoxia, and rapamycin groups. In the hypoxia group, cells were incubated with 1% O2 for 2 h. In the rapamycin group, cells were pretreated with 50 nM rapamycin from *Streptomyces hygroscopicus* (Sigma) for 2 h before hypoxia treatment. Rapamycin, a specific inhibitor of mTOR, induces cell autophagy. After being washed, the cells were incubated with FITC-Annexin V and PI as above. The apoptotic cells were analyzed by flow cytometry.

Statistical analysis. Statistical analysis was performed with SPSS software (version 12.0). Data are presented as means ± SD and were calculated from at least three separate experiments, and each experiment was performed in triplicate. The normality of the data was analyzed by Kolmogorov-Smirnov test. Mean comparisons were analyzed by Student’s t-test of independent samples and one-way analysis of variance with Scheffe’s post hoc multiple-comparison analysis to compare the mean between different groups. The level of significance accepted was *P* < 0.05.

RESULTS

Biologic characteristics of EPCs. By flow cytometric analysis, the frequency of CD34+/VEGFR-2+ cells and CD133+/VEGFR-2+ cells is 0.83% and 0.62% of the mononuclear cells isolated from human cord blood, respectively (Fig. 1A). VEGFR-2, CD133, and CD34 were expressed on EPC membrane (Fig. 1B). The freshly sorted CD34+/VEGFR-2+ EPCs were round or oval. Under induction with VEGF, the cells could proliferate and differentiate toward endothelial cells. At day 10 after induction with VEGF, some cells appeared spindle-like or polygonal (Fig. 1C). Positively sorted CD34+/VEGFR-2+ cells were used in the following experiments.

Effects of autophagy on proliferation and differentiation of EPCs. Cell cycle was analyzed with flow cytometry (Fig. 2A). The proliferation index of the cells in the control group was

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**Fig. 4. Demonstration of the autophagic structures with monodansylcadaverine (MDC) and microtubule-associated protein 1 light chain 3 (LC3) stains.**

**A:** MDC-labeled autophagic structures. After treatment with hypoxia, the cells containing MDC-labeled structures increased. In the 3-MA-treated group, hypoxic cells containing MDC-labeled structures decreased significantly.

**B:** Statistical analysis of the cells containing MDC-labeled structures.

**C:** LC3-positive puncta. The puncta in the cells of hypoxia groups increased. After treatment with 3-MA, the puncta in the hypoxic cells decreased.

**D:** Statistical analysis of LC3-positive puncta. The experiments were repeated three times. *P* < 0.05 and **P** < 0.01 vs. control group; †*P* < 0.05 vs. hypoxia 30 min group; ‡*P* < 0.05 and ‡‡*P* < 0.01 vs. hypoxia 1 h group. Bars, 10 μm.

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24.06 \pm 3.25\%$. After treatment with 3-MA, the proliferation index of the cells decreased to 13.55 \pm 3.71\%. The difference between the two groups was significant (Table 1). By MTT assay, the viability of the cells decreased after treatment with 3-MA. Differences in cell viability at 24, 36, and 48 h after incubation between 3-MA and control groups were significant (Fig. 2B). 3-MA-treated cells did not differentiate into endothelial cells at day 14 after induction with VEGF (data not shown).

Beclin-1 expression of EPCs after hypoxia treatment. By immunostaining, Beclin-1 appeared in a granular and homogeneous pattern and was found almost exclusively in the perinuclear region. Compared with the control cell group, Beclin-1 expression of the cells subjected to hypoxia increased. Beclin-1 expression increased as hypoxic conditions were extended. After pretreatment with 3-MA, Beclin-1 expression of the hypoxic cells was inhibited (Fig. 3A). By RT-PCR analysis, the level of Beclin-1 mRNA expression was significantly higher in hypoxic cells than in the control group. Differences in Beclin-1 mRNA expression among the three hypoxia groups were significant. In the 3-MA-treated group, Beclin-1 mRNA expression was reduced (Fig. 3B).

Changes of autophagic ultrastructures in hypoxia-treated cells. By cytochemical staining, MDC was concentrated in round or oval structures. Compared with the control group, the number of cells containing MDC-labeled autophagic structures in the hypoxic groups was greater. Differences in the number of cells containing MDC-labeled structures among the three hypoxia groups were significant. When cell autophagy was inhibited with 3-MA, the number of cells containing MDC-labeled structures was decreased (Fig. 4, A and B).

Autophagic structures labeled with LC3 immunostaining were round or oval. The number of LC3-positive puncta in the cells in the hypoxic groups was greater than that in the control group. There were significant differences in the number of puncta among the hypoxia groups. In the 3-MA-treated group, the puncta were reduced (Fig. 4, C and D).

Changes of autophagic ultrastructures in hypoxia-treated cells. Autophagic ultrastructures were examined by transmission electron microscopy. Representative autophagic structures in hypoxia-treated cells are shown in Fig. 5. Autophagosome precursors present double-membrane structures that are crescentiform or cuplike (Fig. 5, A and B). Autophagosomes are round or oval double-membrane structures, which encompass electron lucent cytoplasm or dense organelles (Fig. 5C). Autophagosomes with multilayer membranes were observed occasionally (Fig. 5D). In autolysosomes, the lysosome contains an autophagosome that has a single membrane (Fig. 5F). Microautophagy occurred in some hypoxic cells (Fig. 5E). Ratios of the cross-sectional areas of the autophagic structures to that of the cytoplasm in hypoxia groups were significantly higher than in the control group. There were significant differ-

![Fig. 5. Representative autophagic ultrastructures in hypoxic cells. The cells were treated with 1% O2 for 2 h. A: crescentiform autophagosome precursors with double membranes (arrows). B: a cuplike autophagosome precursor enclosing a damaged mitochondrion (arrow). C: an autophagosome with double-membrane structure (arrow). D: an autophagosome (arrow) with multilayer membranes (arrowheads). E: microautophagy. The membrane of the lysosome invaginated and engulfed the cytosol (arrow). F: an autolysosome containing an autophagosome with single membrane (arrow). Bar, 10 \mu m.](http://ajpcell.physiology.org/lookup/doi/10.1152/ajpcell.00296.2012)
Changes in the ratios among the hypoxia groups. After treatment with 3-MA, the ratios of the autophagic structures in the hypoxic cells were decreased (Table 2).

Changes in number of lysosomes after hypoxia treatment. The difference in the number of lysosomes between the control group and the hypoxic 30 min group was not significant. However, compared with the control group, lysosomes in the hypoxic 1 h and hypoxic 2 h groups increased significantly. The number of lysosomes in the hypoxic 2 h group was greater than that in the hypoxic 1 h group. In the 3-MA-treated group, lysosomes were smaller and fewer (Fig. 6).

Changes in apoptosis after inhibition of autophagy. To assess cell death by flow cytometric analysis, cells were stained with FITC-Annexin V and PI. The number of apoptotic cells in the cells treated with hypoxia for 1 h was increased compared with the control group. Compared with the hypoxic group, the apoptotic cells in the 3-MA group increased significantly (Fig. 7, A and B).

By EB/AO staining, the nuclei of normal cells are uniformly green. Early apoptotic cells have shrunken and chromatin-condensed nuclei, while the nuclei of late apoptotic cells are orange-red in color, and the nuclei of necrotic cells appear uniformly red. Early and late apoptotic cells in the hypoxic groups were more frequent than in the control group. After pretreatment with 3-MA, the apoptotic cells in the hypoxic cells increased significantly (Fig. 7C). The numbers of the necrotic cells were 0.1 ± 0.0%, 0.5 ± 0.1%, and 1.3 ± 0.1% in control, hypoxia, and 3-MA groups, respectively.

Effect of enhanced autophagy on inhibition of hypoxia-induced apoptosis. Enhancement of autophagy of the cells was achieved by pretreatment with rapamycin. The apoptotic cells among the cells treated with hypoxia for 2 h increased obviously. Compared with the hypoxic group, the apoptotic cells in the rapamycin-treated group were significantly reduced (Fig. 8). In the control, hypoxia, and rapamycin groups, there were 0.8 ± 0.1%, 2.7 ± 0.5%, and 0.8 ± 0.3% necrotic cells, respectively.

DISCUSSION

In this study, we demonstrate that autophagy is involved in EPC proliferation and differentiation. After basal autophagy was inhibited with 3-MA, proliferation and viability of EPCs were reduced and the cells failed to differentiate into endothelial cells. Basal autophagy is important for maintaining normal cellular homeostasis. Currently, there is growing evidence for a role of autophagy in the control of cell proliferation (47). Following growth factor withdrawal, autophagy increases and cell size declines in the G0/G1 arrested cells (23). Autophagy is strongly inhibited in mitosis cells, and autophagic vacuoles appear after closure of the nuclear envelope in telophase/G1 (11).

After treatment with inducers, autophagic cells increase in S phases, diminish in G2/M phase, and attain maximum values in G1 phase (42). These studies show that autophagy is most active in the G1 and S phases of the cell cycle and inhibited in mitosis. In cell differentiation, recent studies suggest that autophagy is involved in erythropoiesis, lymphopoiesis, and adipogenesis (24). Autophagy promotes the generation and differentiation of induced pluripotent stem cells (46). Blocking autophagy abolishes self-

Fig. 6. Changes in number of lysosomes after hypoxic treatment. Lysosomes were labeled with LysoSensor green, and nuclei were stained with Hoechst 33342. A: micrographs of lysosomes in the cells. Lysosomes are dispersed in the cytoplasm. Bar, 10 μm. B: statistical results of numbers of lysosomes. The numbers of lysosomes in hypoxia 1 h and hypoxia 2 h groups were greater than that in control group. In 3-MA-treated group, the number of lysosomes was reduced significantly. The experiment was repeated for three times. *P < 0.05 and **P < 0.01 vs. control group; †P < 0.05 compared with hypoxia 30 min group; ††P < 0.01 vs. hypoxia 30 min group; #P < 0.05 vs. hypoxia 1 h group.

Table 2. Ratio of cross-sectional area of autophagic structure to that of the cytoplasm

<table>
<thead>
<tr>
<th>Group</th>
<th>Autophagosome Precursor, %</th>
<th>Autophagosomes, %</th>
<th>Autolysosomes, %</th>
<th>Total, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.72</td>
<td>2.05</td>
<td>4.92</td>
<td>7.69</td>
</tr>
<tr>
<td>Hypoxia 30 min</td>
<td>1.57 ± 0.01a</td>
<td>4.17 ± 0.43a</td>
<td>7.93 ± 0.77a</td>
<td>13.67 ± 0.86a</td>
</tr>
<tr>
<td>Hypoxia 1 h</td>
<td>3.11 ± 0.32abc</td>
<td>7.53 ± 0.73abc</td>
<td>10.42 ± 0.84abc</td>
<td>21.06 ± 1.08abc</td>
</tr>
<tr>
<td>Hypoxia 2 h</td>
<td>7.01 ± 0.63bcde</td>
<td>10.72 ± 0.84bcde</td>
<td>14.58 ± 0.95bcde</td>
<td>32.31 ± 1.74bcde</td>
</tr>
<tr>
<td>3-MA</td>
<td>0.33 ± 0.01e</td>
<td>1.02 ± 0.01e</td>
<td>2.31 ± 0.02e</td>
<td>3.66 ± 0.34e</td>
</tr>
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</table>

Values are means ± SD; n = 200 cells/group. *P < 0.05 and **P < 0.01 vs. control group; †P < 0.05 vs. hypoxia 30 min group; ††P < 0.05 and †††P < 0.01 vs. hypoxia 1 h group.
renewal and differentiation of adult stem cells (35). The results of our experiments suggest that autophagy plays an important role in the proliferation and differentiation of stem/progenitor cells. In recent years, the proliferation and differentiation of stem cells in a hypoxia microenvironment have been investigated intensively (40). Most stem/progenitor cells reside in discrete low O₂ niches, which is required to maintain the full differential potential (29). Hypoxia enhances the generation of induced pluripotent stem

Fig. 7. Increase of apoptosis in the cells after pretreatment with 3-MA. The cells were treated with 1% O₂ for 1 h. A: typical quadrantal diagrams of flow cytometric analysis of the apoptotic cells stained with FITC-Annexin V and propidium iodide (PI). The top left, top right, and bottom right plots represent the necrotic cells and the late and early apoptotic cells, respectively. The apoptotic cells in the hypoxia group (6.2%) were more than in control group (2.6%). Compared with the hypoxia group, the apoptotic cells increased significantly in the 3-MA group (12.0%). B: statistical analysis of the apoptotic cells. The experiment was repeated three times. *P < 0.05 and ***P < 0.01 vs. control group; #P < 0.05 vs. hypoxia group. C: quantification of the percentage of the apoptotic cells stained with ethidium bromide and acridine orange (EB/AO). After treatment with 3-MA, the percentage of the apoptotic cells in hypoxia-induced cells increased. The experiment was repeated three times. *P < 0.05 and ***P < 0.01 vs. control group; ###P < 0.01 vs. hypoxia group.

Fig. 8. Decrease of apoptosis in the cells after pretreatment with rapamycin. The cells were treated with rapamycin for 2 h and then incubated with 1% O₂ for 2 h. A: typical quadrantal diagrams of flow cytometric analysis of the apoptotic cells. Compared with hypoxia group, the apoptotic cells (early apoptotic cells in the bottom right plot and late apoptotic cells in the top right plot) in the rapamycin group decreased. B: statistical analysis of the apoptotic cells. The experiment was repeated three times. **P < 0.01 vs. control group; ##P < 0.01 vs. hypoxia group.
cells (48). Low O2 concentration (0.1%) induces the G0 return of CD34+ cells derived from cord blood (15). Differentiation of human embryonic stem cells is markedly reduced under hypoxic conditions (12). Interestingly, autophagy is critical for limiting cell growth and promoting cell survival in times of stress (47). Proliferation and differentiation are the main characteristics of EPC growth and crucial steps for EPC-induced angiogenesis. Therefore, effects of autophagy on proliferation and differentiation of EPCs could be important for angiogenesis of ischemic tissue.

Our experimental data show that levels of autophagy were enhanced in hypoxic cells compared with normoxic cells. During treatment with short-term severe hypoxia (1% O2, 30 min, 1 h, or 2 h), expression of Beclin-1, MDC-, and LC3-positive puncta, and autophagic ultrastructures all increased in a time-dependent fashion. When autophagy of hypoxic cells was inhibited with 3-MA, the number of apoptotic cells increased. Hypoxia-inducible factor 1 (HIF-1), a heterodimeric transcription factor, plays an essential role in the maintenance of oxygen homeostasis (37). HIF-1 mediates adaptive responses to reduced oxygen availability by reduced mitochondrial mass and/or metabolism, which contributes to the protective effects of ischemic preconditioning (28). Hypoxia-induced mitochondrial autophagy requires HIF-1-dependent expression of Beclin-1 and BNIP3 (4, 50). Autophagy removes the damaged mitochondria, thereby reducing reactive oxygen species production and DNA damage and inhibiting the release of proapoptotic factors such as cytochrome c (36). The results in this study provide evidence that autophagy induced by short-term hypoxia protects EPCs, promoting survival and inhibiting apoptosis. Recently, ischemic cell injury experiments showed that mild ischemia leads to induction of autophagy and apoptosis, while moderate or severe ischemia induces both apoptotic and necrotic cell death (22). Autophagic changes of EPCs exposed to more prolonged hypoxia will need further investigation.

Lysosomes are ubiquitous organelles that constitute the primary degradative compartments of the cell, receiving substrates through endocytosis, phagocytosis, or autophagy (34). In our experiments, the lysosomes in hypoxia-induced cells increased as autophagy was enhanced. After treatment with 3-MA, the number and size of lysosomes decreased markedly. Extensive autophagy generates proto-lysosomal tubules and vesicles that extrude from autolysosomes and ultimately mature into functional lysosomes, thereby restoring the full complement of lysosomes (49). Effective biogenesis of the lysosome is implicated in meeting the demand of enhanced cell autophagy in hypoxic conditions.

The present study suggests that the activation of autophagy promotes the resistance of EPCs to hypoxia for survival. Rapamycin in high concentrations inhibits cell proliferation (39). Therefore, low concentrations of rapamycin were used for activating autophagy in this study. Current research focuses on optimization of stem cell transplantation with preconditioning (14). Hypoxic preconditioning of MSCs reduces apoptosis of the implanted cells, enhancing repair of the infarcted myocardium (17). Preculturing under hypoxic conditions prior to transplantation increases motility and improves tissue regenerative potential of MSCs (33). MSCs exposed to low O2 express prosurvival, proangiogenic, and prodifferentiation proteins significantly (8). Biological characteristics of EPCs are different from that of mature endothelial cells and other progenitor/stem cells. By limiting oxygen consumption, the oxygen uptake rate of human EPCs is low in hypoxic culture. Oxygen consumption of human EPCs is similar to that of human umbilical vein endothelial cells and lower than that of human embryonic stem cells and induced pluripotent stem cells (1). Since EPCs may maintain survival in a hypoxic microenvironment by autophagic mechanisms, it may be possible to develop strategies to augment survival and differentiation of homing or transplanted EPCs. According to the results of our experiments, we suggest that the contribution of autophagy to hypoxic preconditioning may be beneficial for adaptation of the transplanted EPCs to the ischemic microenvironment.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
H.-J.W., D.Z., and Y.-Z.T. conception and design of the research; H.-J.W., D.Z., and Y.-Z.T. drafted the manuscript; H.-J.W., D.Z., and Y.-Z.T. acquired the data; H.-J.W., D.Z., and Y.-Z.T. interpreted the results of the experiments; H.-J.W., D.Z., and Y.-Z.T. prepared the figures; H.-J.W. and D.Z. drafted the manuscript; H.-J.W., D.Z., and Y.-Z.T. edited and revised the manuscript; H.-J.W., D.Z., Y.-Z.T., and T.L. approved the final version of the manuscript.

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