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Hypoxia induces the dysfunction of human endothelial colony-forming cells *via* HIF-1α signaling

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ABSTRACT

Endothelial injury is considered as a trigger of pulmonary vascular lesions in the pathogenesis of hypoxic pulmonary hypertension (HPH). Although endothelial colony-forming cells (ECFCs) have vascular regeneration potential to maintain endothelial integrity, hypoxia-induced precise alteration in ECFCs function remains controversial. This study investigated the impact of hypoxia on human ECFCs function *in vitro* and the underlying mechanism. We found that hypoxia inhibited ECFCs proliferation, migration and angiogenesis. Compared with no treatment, the expression of hypoxia inducible factor-1α (HIF-1α) in hypoxia-treated ECFCs was increased, with an up-regulation of p27 and a down-regulation of cyclin D1. The over-secreted vascular endothelial growth factor (VEGF) was detected, with the imbalanced expression of fetal liver kinase 1 (flk-1) and fms related tyrosine kinase 1 (flt-1). Hypoxia-induced changes in ECFCs could be reversed by HIF-1α inhibitor KC7F2. These data suggest that HIF-1α holds the key in regulating ECFCs function which may open a new perspective of ECFCs in HPH management.

1. Introduction

Pulmonary hypertension (PH) is featured as the increase in pulmonary artery pressure and elevation of pulmonary vascular resistance, leading to right ventricular hypertrophy and death ultimately (Long et al., 2015). As an important type of PH, hypoxic PH (HPH) is generally observed with chronic exposure to sustained or intermittent hypoxia (Galie et al., 2016). Pulmonary artery endothelial cells (PAECs) have gained sufficient attention for their decisive roles in the progression of pulmonary vascular remodeling. PAECs exhibit abnormal proliferation and apoptosis resistance, as well as a freak control of pulmonary artery smooth cells (PASMCs) when hypoxia occurs (Gao et al., 2016; Kourembanas et al., 1991; Stenmark et al., 2015). Many efforts are directed into the protection of endothelial cell integrity, but preliminary evidence of clinical data implies poor prognosis. Therefore, there is an urgent need for effective therapeutic options for managing HPH.

Endothelial progenitor cells (EPCs), first isolated from adult peripheral blood in 1997 (Asahara et al., 1997), are regarded as the precursor of ECs. They exhibit endothelial features, but not identical to ECs. EPCs have more aggressive proliferative potential with the capability to form new vessel networks (Miller-Kasprzak and Jagodzinski, 2007; Recchioni et al., 2016). The number of circulating EPCs in patients with PH is much lower than that in normal controls (Hansmann et al., 2011). Recently, EPCs have been reported as biomarkers of PH for predicting the prognosis. Although EPCs-based cell therapy has promising potential in PH treatment (Yang et al., 2013), transfer of EPCs fail to reverse disease progression in mice for the poor rescue of endothelial integrity (Marsboom et al., 2008). As a unique subtype of EPCs, endothelial colony forming cells (ECFCs) have been well documented to belong to endothelial lineage (Basile and Yoder, 2014). Despite of their capacities to maintain the re-endothelialization of damaged vessels, hypoxia-induced precise alteration in ECFCs function still remains controversial (Avouac et al., 2008; Decaris et al., 2009; Dincer, 2015). The present study focused on the underlying mechanism in the changes of ECFCs function with an exposure to hypoxia.

Hypoxia inducible factor-1α (HIF-1α), a critical responsive element to hypoxia, is closely implicated in vascular remodeling in HPH (Hubbi and Semenza, 2015). Accumulating data have shown that the expression of HIF-1α is augmented in HPH rats; mice lack of a single HIF-1α allele exhibit attenuated vascular remodeling in lungs and develop a

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milder form of HPH (Ball et al., 2014). Additionally, HIF-1α activation contributes to the dysfunction of pulmonary cells, such as excessive proliferation and migration of PASMCs (Li et al., 2016), altered permeability of endothelium, over-production of pro-inflammatory cytokines, and an imbalance between vasoconstrictors and vasodilators of PAECs (Bryant et al., 2016). Although extensive efforts have been devoted to investigating the role of HIF-1α in hypoxic response, little is known about its contribution to regulating ECFCs in hypoxia.

In the present study, we investigated the effects of hypoxia on ECFCs proliferation, migration and angiogenesis *in vitro*. Molecular biological analysis was conducted to uncover the role of HIF-1α in modulating ECFCs function in hypoxic conditions.

2. Materials and methods

2.1. Chemicals

A specific HIF-1α inhibitor, KC7F2 was purchased from Selleck (Selleck, Texas, USA). KC7F2 was diluted with dimethyl sulphoxide (DMSO). The concentration of DMSO was controlled below 0.1% (vol/vol), and did not affect the biological viability of the culture cells.

2.2. Isolation and phenotype characterization of ECFCs

Blood samples were collected from healthy volunteers recruited from the First Affiliated Hospital of Nanjing Medical University (Nanjing, Jiangsu, China). The inclusion criteria were: age between 18 and 60 years old; either sex; clinically healthy; and voluntary consent to participate in the study. Exclusion criteria were: age less than 18 years old or more than 60 years old; clinical evidence of acute or chronic illness; past history of smoking; and refusal to consent to study participation. The study was approved by the First Affiliated Hospital of Nanjing Medical University. Informed consent was obtained from all volunteers before sample collection. ECFCs were obtained from adult peripheral blood *via* density gradient centrifugation in a Ficoll-Paque (Sigma-Aldrich, Saint Louis, USA) gradient as previously described (Wu et al., 2017). Isolated ECFCs were cultured in endothelial basal medium-2 (EBM-2) (Lonza, Walkersville, USA) with 10% fetal bovine serum (FBS), epidermal growth factor (EGF), VEGF, basic fibroblast growth factor (FGF), recombinant insulin-like growth factor-1 (IGF-1), gentamicin/amphotericin-B, ascorbic acid and heparin (Lonza, Walkersville, USA). The ECFCs used in the study were less than eighth passages.

The cultured cells were tested for uptake of both DiI-labeled acetylated low-density lipoprotein (DiI-ac-LDL) (Thermo fisher scientific, USA) and FITC-labeled Ulex europaeus agglutinin-1 (FITC-UEA-I) (Sigma-Aldrich, Saint Louis, USA). Immunofluorescence staining was used for additional molecular identification. Briefly, after fixation and blocking, cells were stained with the primary antibodies CD31 (Santa Cruz, CA, USA) and VE-cadherin (Abcam, Cambridge, UK) at 4 °C overnight. Then, the cells were incubated with Alexa Fluor 488 donkey anti-goat IgG (Thermo fisher scientific, Waltham, USA). The nuclei were counterstained with 4′, 6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Saint Louis, USA). Cells were visualized using an inverted fluorescent microscope (DM2500, Leica, Wetzlar, Germany).

2.3. Hypoxia experiments

The oxygen (O_2) microenvironment was manipulated using a commercially available modular incubator chamber (Hua Xi Electronics Technetronic Company, Changsha, China). Cells were cultured under normoxia (94% air and 5% CO_2) or hypoxia (93% N_2, 5% CO_2, and 2% O_2, or 94% N_2, 5% CO_2, and 1% O_2). When the needed O_2 level was reached, the chamber was sealed, maintaining a gas mixture at 37 °C in a humidified atmosphere. For further clarifying the role of HIF-1α in hypoxia, cells were stimulated with 1% O_2 in the presence of KC7F2 (10 nM) for 24 h.

2.4. Cell proliferation assays

The cell counting kit-8 (Dojindo Molecular Technologies, Kumamoto, Japan) assay was applied to detect ECFCs viability. In general, ECFCs at passage 4, 5, 6 and 7 from the same blood donor were seeded in 96-well at a density of 1×10^4/well overnight. Then, the cells were exposed to different O_2 level, or placed under hypoxia (1% O_2) for indicated time in the presence of absence of the HIF-1α inhibitor. Cells were incubated in 10% CCK-8 solution for an additional 4 h at 37 °C according to the manufacture's protocol. Optical density (OD) was measured on a microplate reader (Thermo Scientific, CA, USA). The experiment was repeated at least three times in three duplications from each group under identical experimental conditions.

ECFCs proliferative potential was additional assessed by the Cell-Light™ EdU (5-ethynyl-2′-deoxyuridine) imaging detecting kit (Ribobio, Guangzhou, China). All the procedures were conducted following the manufacturer's protocol. In brief, ECFCs at passage 4–7 from the same blood donor were seeded in 24-well culture dishes, allowing attaching overnight. Cells were exposed to hypoxia as stated above. Then, ECFCs were incubated with 50 μmol/L EdU for 4 h before fixation, permeabilization, EdU staining and nucleus staining. Cells were observed by fluorescence microscopy (Lescia, Wetzlar, Germany). The percentage of EdU-positive cells (red) to total cells (blue) was calculated as the proliferation rate of ECFCs in five random high-power fields per well.

2.5. Cell migration assay

Transwell migration chamber assay was applied to assay ECFCs migration as previous stated (Wu et al., 2017). ECFCs at passage 4, 6 and 7 from the same blood donor were resuspended at 1×10^4 cells/ml in serum-free medium. 100 ul cell suspension were added in the upper chamber of a 24-well Transwell (Corning, New York, USA), while 600 ul of EBM-2 medium containing 10% FBS was added in the lower compartment of the chamber. The chambers were placed under normal or hypoxic conditions as described above. Then, ECFCs attached on the top membrane were wiped off with a cotton swab. Cells that migrated to the lower side of the transwell membrane were fixed with 4% formaldehyde, and stained with 5% crystal violet. Five vision fields were randomly counted using a phase contrast microscope (Nikon, Tokyo, Japan).

2.6. In vitro tube formation on matrigel plates

ECFCs angiogenesis was assayed when culturing in varied O_2 levels for indicated time as stated previously. 10 ul matrigel (Becton-Dickinson, San Diego, USA) was pipetted into a u-slide angiogenesis plate (ibidi, Martinsried, German) for pre-incubation at 37 °C for 1 h. Then, 50 ul ECFCs suspension at the passage 4–7 from the same blood donor resuspended at the concentration of 1.5×10^4 cells per ml were seeded on matrigel in complete medium. Cells were then incubated in either normoxia or hypoxia with or without the HIF-1α inhibitor to allow *in vitro* angiogenesis. Randomly, five images of tube were captured for each well. The length of complete tubes per image was measured to determine ECFCs angiogenesis by Image-Pro Plus.

2.7. Cell cycle analysis

Cell cycle assays were performed using flow cytometry *via* propidium iodide (PI) staining. Briefly, cells at the passage 4, 5 and 6 from the same blood donor were cultured in EBM-2 without serum and growth factors for 24 h to allow synchronizing. When re-placed in EBM-2 with serum, ECFCs were incubated in normoxia or hypoxia (1% O_2). Then, cells were harvested, washed twice in PBS, and resuspended in 70% (v/v) pre-cooled ethanol for 24 h. Then, cells were incubated with