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Hydrogen peroxide induced impairment of endothelial progenitor cell viability is mediated through a FoxO3a dependant mechanism☆

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ABSTRACT

Objectives: Increased oxidative stress has been suggested to contribute to the functional impairment of endothelial progenitor cells (EPCs). The Forkhead box O transcription factors (FoxOs) are critical regulators involved in various cellular processes including cell apoptosis. Here, we investigated whether FoxOs are required in oxidative stress induced EPC apoptosis.

Methods and results: EPCs were cultured from cord blood derived mononuclear cells and treated with hydrogen peroxide (H_2O_2) for induction of oxidative stress. Incubation with H_2O_2 dose dependently reduced viability and increased apoptosis in EPCs. Western blotting showed that EPCs predominantly expressed FoxO3a and the expression was markedly increased upon H_2O_2 treatment. Transduction with adenoviral vectors expressing either a wide-type or a non-phosphorylatable, constitutively active mutant of FoxO3a led to further increased apoptosis of EPCs after H_2O_2 treatment. Conversely, FoxO3a silencing rescued EPCs from these H_2O_2 induced deleterious effects. Overexpression of FoxO3a also increased the level of the pro-apoptotic protein Bim, whereas FoxO3a silencing downregulated H_2O_2 induced Bim expression. Furthermore, Matrigel assay demonstrated that FoxO3a overexpression significantly impaired the tube forming ability of EPCs, whereas its silencing completely protected EPCs from H_2O_2 induced decrease of capillary formation.

Conclusions: These data suggest that oxidative stress induced impairment of EPC survival is mediated through a FoxO3a dependant mechanism, possibly by transcriptional regulation of Bim. Our data indicate FoxO3a as a potential therapeutic target for improvement of EPC number and function in patients with ischemic heart disease.

Introduction

Considerable evidence has suggested that endothelial progenitor cells (EPCs) importantly participate in postnatal neovascularization and re-endothelialization of damaged endothelium (Asahara et al., 1999; Griese et al., 2003; Zampetaki et al., 2008). EPCs can be isolated from bone marrow as well as peripheral and cord-blood derived mononuclear cells (Kawamoto et al., 2001; Murohara et al., 2000). Either transplantation or endogenous mobilization of EPCs has been shown to improve regional blood flow as well as functional recovery in animal models of hindlimb ischemia or myocardial infarction (Kawamoto et al., 2003; Takahashi et al., 1999). However, clinical studies demonstrate that the number and function of EPCs are adversely affected by classical risk factors for coronary artery disease (CAD) (Vasa et al., 2001; Werner and Nickenig, 2006), thus potentially limiting their efficacy to promote neovascularization in patients with ischemic heart disease.

Oxidative stress, resulting from imbalance of reactive oxygen species (ROS) generation and detoxification, is a hallmark of CAD and its related risk factors such as aging, smoking, hypertension, and diabetes (Cai and Harrison, 2000). Prior studies have shown that ROS directly leads to endothelial dysfunction and contribute to the progression of atherosclerosis (Cai and Harrison, 2000; Taniyama and Griendling, 2003; Touyz, 2004). Although EPCs have been reported to be uniquely equipped with intrinsic cellular machinery to detoxify ROS and more resistant to oxidative stress as compared with mature endothelial cells, impairment of EPCs survival and function has been observed under conditions of oxidative stress induced by hydrogen peroxide (Dernbach et al., 2004; He et al., 2004; Ingram et al., 2007). It is probable that oxidative stress is, at least in part, a common cause of EPCs dysfunction in patients with CAD. The molecular mechanisms underlying the deleterious effects of oxidative stress on EPCs have not been completely understood.

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The forkhead hemeobox type O (FoxO) transcription factors FoxO1, FoxO3a and FoxO4 are critical regulators involved in various cellular processes including cell survival and apoptosis (Calnan and Brunet, 2008; Puig and Mattila, 2011). Transcriptional activity of FoxOs primarily depends on its nuclear localization. In the presence of serum and growth factors, the survival kinase Akt is phosphorylated, which in turn phosphorylates FoxOs, leading to nuclear exclusion, cytoplasm retention and inactivation of FoxOs (Accili and Arden, 2004; Zhang et al., 2011). Conversely, oxidative stress can induce re-localization of FoxOs from the cytoplasm to the nucleus and activate FoxOs target genes, including the pro-apoptotic gene Bim, with subsequent cell apoptosis (Dijkers et al., 2000; Storz, 2011). This nuclear translocation of FoxOs is considered to be part of the mechanism whereby oxidative stress impairs cell viability. In the present study, we examined whether FoxOs transcription factors are required in oxidative stress induced EPC apoptosis.

Materials and methods

EPC isolation and cultivation

EPCs were cultured from human cord blood derived mononuclear cells (MNCs) by density gradient centrifugation with Histopaque-1077 (Sigma). After washing steps with phosphate buffered saline (PBS), isolated cells were resuspended in EC-basal medium (EBM-2) supplemented with EGM-2MV Single Quots (Clonectics) and 5% fetal calf serum (FCS) and seeded on fibronectin pre-coated culture plates. After 3 days, non-adherent cells were removed and adherent cells were maintained in fresh medium. Thereafter, medium change was conducted every 3 days. Cell colonies were trypsinized and passaged when necessary. Third to fourth passage cells were used for all subsequent experiments. To induce oxidant stress, cells were incubated with different concentrations of hydrogen peroxide (Sigma).

For determination of an EPC phenotype, cells were characterized by expression of endothelial marker proteins vWF (Antibody Corporation), CD31 (Antibody Corporation) and KDR (Santa Cruz Biotechnology) and by dual staining for acetylated low density lipoprotein (Dil-acLDL, Molecular Probes) uptake and UEA-1 binding. Besides, in vitro tube formation assay was also used as a functional property of EPCs.

Adenoviral construction and transduction

The human FoxO3a triple mutant and wide-type sequence cDNA (purchased from Genecopoeia USA) was subcloned into a shuttle vector pShuttle-CMV-EGFP, which contains green fluorescent protein under the control of a separate cytomegalovirus (CMV) promoter. The triple mutant form of FoxO3a has been described elsewhere (Skurk et al., 2004). It is not phosphorylatable by Akt because the three conserved Akt phosphorylation sites, Thr-32, Ser-253 and Ser-315 were replaced by alanine residues. For RNA interference, the hairpin structure cDNA was PCR amplified from a confirmed siRNA sequence that specially inhibits FoxO3a expression (Sigma, TRCN0000010335), and was subcloned into the shuttle vector pShuttle-Basic-EGFP. These recombinant shuttle plasmids were linearized and transformed into *Escherichia coli* bacteria to recombine with the adenoviral backbone plasmid pAdeno. The resultant recombinant adenoviral plasmid was transfected into the packaging cell line (293 cells) for production and amplification of adenovirus. Adenoviral vector expressing only the GFP transgene (Ad-GFP) was generated by the same system and was used as control. All viral constructs were purified by CsCl ultracentrifugation. Titration was performed by means of the plaque assay.

For transduction, EPCs at approximately 80% confluence in six-well culture plates were washed with PBS and transduced with the indicated recombinant adenoviral vectors at a multiplicity of infection of 40. After 2 h of incubation, cells were washed and fresh medium was applied. Cells were harvested at 24 h of transduction for subsequent experiments as described below.

MTS assay

For evaluation of EPC viability, MTS assay (Promega) was performed according to the manufacturer's instructions. Cells were detached using 0.25% Trypsin (Gibco), washed and harvested by centrifugation. Then, equal number of cells was reseeded in 96-well plates in triplicate with complete EGM-2 in the presence or absence of H_2O_2. After 24 h of incubation, 20 μl MTS/PMS solution was added to each well for 4 h, and light absorbance at 490 nm was measured using an ELISA plate reader. Three independent experiments were conducted.

FACS analysis of cell apoptosis

Equal number of EPCs was seeded in 24-well culture plates in triplicate with complete EGM-2 in the presence or absence of H_2O_2. After incubation for 24 h, apoptotic cell death was determined using an Annexin-V FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, adherent and nonadherent cells were collected and washed with PBS and then resuspended in Binding Buffer. Cells were stained with Annexin V-FITC diluted 1:10 for 15 min at room temperature in the dark. After washing steps and staining with PI, apoptotic cells were quantified by flow cytometry. Three independent experiments were performed.

Matrigel assays

Matrigel basement membrane matrix (BD Biosciences) was used for assessment of in vitro tube forming ability of EPCs as previously described (Chen et al., 2009). In brief, the same number of cells was seeded in each well of 96-well culture plates precoated with 30 μl Matrigel in complete EGM-2 with or without H_2O_2 treatment. Twenty-four hours following incubation, wells were observed by visual microscopy for capillary-like structures and images were captured. Four randomly selected fields were evaluated by visual inspection for enumeration of closed network units as areas enclosed by a continuous network of capillary vessels. Three independent experiments were performed and each experiment was performed in triplicate.

Western blot

Whole cell proteins extracts were prepared using standard protocol. For some experiments, the nuclear fractions were separated by use of a commercially available kit according to the manufacturer's instructions (Pierce). The concentrations of the proteins were determined by BCA protein assay (Invitrogen). Equal quantity of protein samples was separated on SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Immunoblotting was performed by the use of primary antibodies against FoxO1, FoxO3a, FoxO4, phospho-FoxO3a, Bim, and phospho-Akt (all from Cell Signaling Technology), followed by incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson Laboratories) at appropriate dilutions and detected by ECL detection reagents (Millipore, Billerica, MA). Anti-β-actin antibody (Sigma, MO, USA) was used as loading control. Three independent experiments were performed.

Statistical analysis

Results are expressed as the mean ± SE. Comparisons between groups were analyzed by one way ANOVA or paired Student's *t* test when appropriate. A probability value of $\leqq 0.05$ was considered to be statistically significant. All analyses were performed with SPSS 14.0 software.