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Clonogenic Endothelial Progenitor Cells Are Sensitive to Oxidative Stress

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ABSTRACT

Endothelial progenitor cells (EPCs) circulate in the peripheral blood and reside in blood vessel walls. A hierarchy of EPCs exists where progenitors can be discriminated based on their clonogenic potential. EPCs are exposed to oxidative stress during vascular injury as residents of blood vessel walls or as circulating cells homing to sites of neovascularization. Given the links between oxidative injury, endothelial cell dysfunction, and vascular disease, we tested whether EPCs were sensitive to oxidative stress using newly developed clonogenic assays. Strikingly, in contrast to previous reports, we demonstrate that the most proliferative EPCs (high proliferative potential-endothelial colony-forming cells and low proliferative potential-endothelial colony-forming cells) had decreased clonogenic capacity after oxidant treatment. In addition, EPCs exhibited increased apoptosis and diminished tube-forming ability in vitro and in vivo in response to oxidative stress, which was directly linked to activation of a redox-dependent stress-induced kinase pathway. Thus, this study provides novel insights into the effect of oxidative stress on EPCs. Furthermore, this report outlines a framework for understanding how oxidative injury leads to vascular disease and potentially limits the efficacy of transplantation of EPCs into ischemic tissues enriched for reactive oxygen species and oxidized metabolites. STEM CELLS *2007;25:297–304*

INTRODUCTION

Accumulating evidence suggest that endothelial progenitor cells (EPCs) circulate in peripheral blood and home to sites of neovascularization, including ischemic tissues and tumor microenvironments [1–7]. From these reports, intense effort has focused on defining the role of EPCs in restoring damaged vascular endothelium and on translating these experimental observations into human clinical trials for repair of vascular injury and/or ischemic tissues [2, 8]. However, the scientific rationale for the transplantation or mobilization of autologous EPCs for vascular repair has been complicated by recent studies, which demonstrate that marrow-derived EPCs may play a minimal role or no role in new blood vessel formation [9–11].

The tissue microenvironment after ischemia is characterized by excessive production of reactive oxygen species (ROS) and oxidized metabolites. Prior studies have demonstrated that oxidative stress directly contributes to endothelial dysfunction and vascular disease [9–15]. These observations suggest that EPCs, in contrast to mature endothelial cells, are uniquely equipped with the cellular machinery to resist ROS-driven cytotoxicity if they are active participants in vascular repair in ischemic tissues. Given the increasing evidence that several methods used to harvest EPCs isolate myeloid progenitors that give rise to angiogenic macrophages and not cells that can form de novo vessels in vivo [16–19], it is imperative to test whether EPCs are sensitive to oxidative stress similar to other progenitor cell types.

Some studies have suggested that EPCs may in fact be resistant to oxidative stress. Limitations of these experiments were the use of cell populations composed of macrophages and monocytes, which are known to be resistant to oxidative stress, and failure to use clonogenic assays to rigorously test the effect of oxidative stress on EPC function [20, 21]. Currently, there is no uniform definition of an EPC, and EPCs are primarily defined by expression of cell-surface antigens (reviewed in [8]). A hallmark of many stem and progenitor cells is their ability to give rise to numerous differentiated progeny and provide sufficient cells for tissue homeostasis. Based on this paradigm, we recently reported the development of a single-cell deposition assay to define a novel hierarchy of EPCs based on their proliferative and clonogenic potential [22]. Given the potential importance of EPCs in the vascular repair of ischemic tissues and the development of clinical protocols to either directly administer or mobilize EPCs into tissues enriched for oxidized metabolites, we directly tested the effect of oxidative stress on purified EPCs using newly developed clonogenic assays.

MATERIALS AND METHODS

Culture of Human Umbilical Vein Endothelial Cells

Cryopreserved human umbilical vein endothelial cells (HUVECs) were obtained from Cambrex (Walkersville, MD, http://www.cambrex.com). Cells were seeded in 75-cm^2 tissue culture flasks

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precoated with type I rat tail collagen in complete endothelial cell medium (EBM)-2 (Cambrex) and cultured as previously described.

Buffy Coat Preparation of Blood Samples

Peripheral blood from healthy adult volunteers (four male, four female) and umbilical cord blood from eight healthy newborns (38–40 weeks gestational age; four males and four females) was collected as previously described [22]. The Institutional Review Board at the Indiana University School of Medicine approved all protocols, and informed consent was obtained from adult donors. Mononuclear cells (MNCs) were obtained from blood samples by gradient centrifugation over Histopaque 1077 (ICN, Costa Mesa, CA, http://www.icnbiomed.com) and washed with EBM-2 medium supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT, http://www.hyclone.com), 2% penicillin/streptomycin (Invitrogen, Grand Island, NY, http://www.invitrogen.com), and 0.25 μg/ml amphotericin B (Invitrogen) as previously described [22].

Culture of EPCs

Buffy coat MNCs were resuspended in endothelial growth medium (EGM)-2 medium (Cambrex) supplemented with 10% FBS, 2% penicillin/streptomycin, and 0.25 μg/ml amphotericin B (complete EGM-2 medium). Cells were seeded onto six-well tissue culture plates (5×10^7 cells per well) precoated with type I rat tail collagen (BD Biosciences, Bedford, MA, http://www.bdbiosciences.com) and incubated at 37°C with 5% CO_2 as previously described [22]. Medium was changed daily for 7 days and then every other day until first passage. Once confluent, EPCs were trypsinized, resuspended in complete EGM-2 medium, and plated onto 75-cm^2 tissue culture flasks coated with type I rat tail collagen. EPC monolayers were passaged after becoming 90%–100% confluent.

Colony-Forming Assays

Early passage EPCs (passages 2–3) were seeded in six-well plates precoated with type I rat tail collagen (500 cells per well). The following day, cells were either left untreated or treated with increasing H_2O_2 concentrations in triplicate for 6 days. On day 7 after seeding the cells onto six-well plates, colonies were scored by visual inspection with an inverted microscope (Olympus, Lake Success, NY, http://www.olympus-global.com) under ×40 magnification.

To assess the colony-forming ability of single EPCs, single EPCs were placed into each well of a 96-well tissue culture plate precoated with type I rat tail collagen using a FACSAria Sorter (Becton Dickinson, San Jose, CA, http://www.bd.com). Cells were adhered overnight before H_2O_2 treatment for 13 days. On day 14 after placing the cells into each well of a 96-well tissue culture plate, wells were examined for colony formation using an inverted microscope under ×100 magnification. To quantitate the frequency of dividing single endothelial cells (ECs), wells that had two or more ECs were scored. To enumerate cells per well, cells were counted by visual inspection with an inverted microscope (<500 cells), or cells were trypsinized and counted with a hemacytometer (>500 cells). To ensure that self-renewal capacity correlated with colony size (>2,000 cells), colonies were replated into secondary cultures as previously described [22, 23].

EPC Growth Kinetics

Cord blood-derived EPCs that were either untreated or treated with increasing H_2O_2 concentrations were grown to 90% confluence in six-well plates before monolayers were passaged. At each passage, viable cells were enumerated by trypan blue exclusion for calculation of growth kinetics and cumulative population doubling levels (CPDLs). Population doublings (PDs) occurring between passages was calculated according to the following equation: $PD = \log_2 (C_H/C_S)$, where C_H is viable cells at harvest and C_S is cells seeded. The sum of all previous PDs determined the CPDL at each passage, as previously described [22].

Apoptosis

EPCs and HUVECs were either untreated or pretreated with 1 mM *N*-acetylcysteine (NAC) for 1 hour prior to culturing with 200 μM H_2O_2 for 24 hours. Adherent and nonadherent cells were collected, and apoptosis was examined using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay per the manufacturer's recommendations (Roche Diagnostics, Indianapolis, IN, http://www.roche-applied-science.com). Conditions were conducted in triplicate, and at least 100 cells were scored per replicate, as previously described [24, 25].

Western Blotting

For immunodetection of active apoptosis signal-regulating kinase 1 (ASK1), EPCs were either untreated or treated with 200 μM H_2O_2 for 5 minutes before lysing whole cell extracts. A phospho-ASK1 (Thr 845 site; Cell Signaling, Beverly, MA) antibody was used at a 1:500 dilution, and the secondary antibody, anti-rabbit-horseradish peroxidase (HRP) (Amersham Biosciences, Piscataway, NJ, http://www.amersham.com) was used at a 1:3,000 dilution before visualizing by chemiluminescence. To examine the expression of antioxidant proteins, whole cell extracts were obtained from adult EPCs, cord blood EPCs, and HUVECs. Mouse monoclonal antibodies recognizing endothelial nitric oxide synthase (clone 3; BD Biosciences), manganese superoxide dismutase (clone 19; BD Biosciences), glutathione peroxidase (clone 347; Calbiochem), and catalase (clone CAT-505; Sigma-Aldrich, St. Louis, http://www.sigmaaldrich.com) were used at 1:200, 1:200, 1:100, and 1:200 dilutions, respectively. An anti-mouse-HRP (Amersham Biosciences) was used at a 1:3,000 dilution before detecting by chemiluminescence. Densitometric analysis of individual bands was conducted using NIH Image software. Mean arbitrary densitometry units were calculated from the three experiments to demonstrate statistical significance. To document equal protein loading, membranes were probed with a β-actin antibody (Sigma-Aldrich).

Retroviral Transduction

Similar to previous studies [25], a transient transfection system was used to obtain high titer ecotropic retroviral supernatants for MigR1 (control retrovirus encoding enhanced green fluorescence protein [EGFP] only) and MigR1-ASK1-K709M (retrovirus encoding a hemagglutinin [HA]-tagged catalytically inactive, dominant-negative ASK1 cDNA and EGFP). To obtain GALV-pseudotyped retroviral supernatants, PG13 packaging cells were plated onto Petri dishes precoated with 2 $\mu g/cm^2$ fibronectin fragment (CH-296; Takara, Otsu, Japan, http://www.takara.co.jp) before transducing with ecotropic MigR1 or MigR1-ASK1-K709M supernatants in the presence of 5 μg/ml polybrene. Transduced PG-13 cells were enriched for $EGFP^+$ cells using a FACSAria sorter. GALV-pseudotyped MigR1 and MigR1-ASK1-K709M supernatants were collected and stored as previously described [25, 26].

Early passage cord blood-derived EPCs (passages 1–2) were transduced on Petri dishes coated with 2 $\mu g/cm^2$ fibronectin fragment (CH-296). Cells were transduced with retroviral supernatant diluted 3:1 with complete EGM-2 in the presence of 8 μg/ml polybrene for 3 days. Forty-eight hours later, cells were sorted for EGFP expression using a FACSAria sorter as previously described [22, 23]. Western blotting was conducted to ensure expression of ASK1-K709M using primary antibodies against either HA or total ASK1 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, http://www.scbt.com). Transduced EPCs were then treated with 200 μM H_2O_2 and either examined by TUNEL using a tetramethylrhodamine (TMR) fluorochrome (Roche) or plated in Matrigel (BD Biosciences) assays as described.

Matrigel Assays

EPCs transduced with either MigR1 or MigR1-ASK1-K709M were treated with 200 μM H_2O_2 for 1 hour, and Matrigel assays conducted as previously described [22]. Cells were seeded at 4,000 to 5,000 cells per well in 96-well tissue culture plates coated with 30 μl of Matrigel (BD Biosciences). Wells were observed over 5 days by visual microscopy for capillary-like formation. At 24 hours, phase contrast images were collected using a Zeiss Axiovert 2 inverted microscope with a ×5 CP-ACHROMAT/0.12 NA objective (Carl Zeiss, Jena, Germany, http://www.zeiss.com). Images were acquired using a SPOT RT color camera (Diagnostic Instru-

ments, Sterling Heights, MI, http://webstore.diaginc.com/) with the manufacturer's software. Total vessel length per well was quantitated from captured images using NIH imaging software (http://rsb.info.nih.gov/ij/). Closed network units were enumerated by visual inspection as areas enclosed by a continuous network of capillary vessels. Each experiment was performed in triplicate.

Xenograft EPC Transplantation

Cellularized gel implants were cast as previously described with minor modifications [27]. Cord blood EPCs were either untreated or treated with 200 μM H_2O_2 for 1 hour before suspending (2×10^6 cells per ml) in 1.5 mg/ml rat-tail collagen I, 100 ng/ml human fibronectin (Chemicon, Temecula, CA, http://www.chemicon.com), 1.5 mg/ml sodium bicarbonate (Sigma-Aldrich), 25 mM HEPES (Cambrex), 10% FBS, and 30% complete EGM-2 in EBM-2. The cell suspensions were placed in a 12-well tissue culture dish (1 ml per well) for 30 minutes at 37°C for polymerization. The gels were then covered with complete EGM-2 for overnight incubation. Gels were implanted the following day into three anesthetized non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. The right flank of each mouse was implanted with untreated cells, and the left flank of each mouse was implanted with H_2O_2-treated cells. Fourteen days after transplantation, the grafts were excised and analyzed for vessel formation by immunohistochemistry. All experiments were approved by the Indiana University Laboratory Animal Research Center.

Immunohistochemistry

All reagents for immunohistochemistry studies were purchased from DAKO (Carpenteria, CA, http://www.dako.com) unless otherwise specified. For anti-human CD31 staining, formalin-fixed, paraffin-embedded tissue sections were deparaffinized and immersed in a retrieval solution for 20 minutes at 95°C–99°C. Slides were incubated at room temperature with anti-human CD31 (clone JC70A) for 30 minutes followed by successive 10-minute incubations with LSAB2 link-biotin and streptavidin-HRP and then developed with diaminobenzidine solution for 5 minutes. The slides were dehydrated and mounted using Cytoseal (Richard-Allan Scientific, Kalamazoo, MI, http://www.rallansci.com). Enumeration of perfused vessels staining positively for anti-human CD31 was performed by visual inspection under ×100 magnification.

Results

Cord Blood and Adult EPCs Are Highly Sensitive to Oxidant Stress

To test whether EPCs are sensitive to oxidant stress, cord blood EPCs were cultured with increasing H_2O_2 concentrations in clonogenic progenitor assays. These studies demonstrated a significant reduction in EPC colony formation at 100 and 200 μM H_2O_2 concentrations compared with untreated controls, with 60% and 80% decreases in colony formation, respectively (Fig. 1A). Similar results were obtained when MNCs were treated with H_2O_2 before culturing for primary EPC colonies (Fig. 1B), suggesting an intrinsic sensitivity of circulating EPCs to oxidant stimuli.

On a single-cell level, cord blood EPCs display significant heterogeneity in proliferative capacity; similar to hematopoietic progenitors, where a hierarchy of primitive to more differentiated progenitors can be cultured from the same cell population. Given this similarity, we previously established an analogous nomenclature to describe EPCs formed in a single-cell deposition assay [8, 22, 23]. The high proliferative potential-endothelial colony-forming cell (HPP-ECFC) forms a colony that contains >2,000 cells with self-renewal potential, defined as the ability to form secondary colonies upon replating. Low proliferative potential-endothelial colony-forming cell (LPP-ECFC) colonies have 51 to 2,000 cells and no self-renewal capacity,

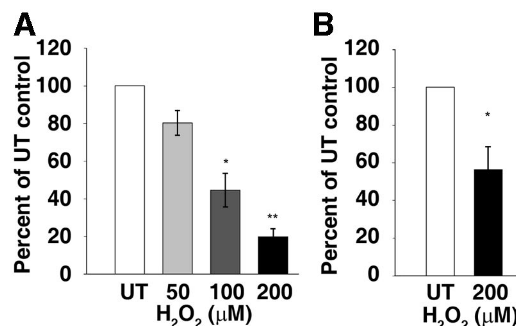

Figure 1. Colony-forming potential of cord blood-derived endothelial progenitor cells (EPCs) and cord blood mononuclear cells (MNCs) following H_2O_2 treatment. **(A):** Percentage of cord blood-derived EPCs forming colonies or clusters 7 days after H_2O_2 treatment. Results represent the mean ± SEM ($n = 3$; *, $p < .03$ and **, $p < .003$ by Student's paired *t* test). **(B):** EPC colony formation following treatment of MNCs with 200 μM H_2O_2. Results represent the mean ± SEM ($n = 3$; *, $p < .02$ by Student's paired *t* test). Abbreviation: UT, untreated.

whereas colonies composed of 2 to 50 cells are considered EC clusters. To evaluate the effects of oxidant stress on a single-cell level, we sorted single EPCs in 96-well tissue culture plates before treating with H_2O_2 and assessing colony formation. The proportion of untreated single cells that divided at least once was similar to previous studies (53.3 ± 8.0%; $n = 3$). H_2O_2 treatment resulted in a dose-dependent decrease in overall colony formation (Fig. 2A), supporting a significant loss of clonogenic EPCs. When colony distribution was enumerated, a profound reduction in HPP-ECFCs at 100 and 200 μM H_2O_2 was detected, whereas LPP-ECFCs were only reduced at the highest H_2O_2 dose evaluated (Fig. 2B). These data suggest that the most primitive EPCs are exquisitely sensitive to H_2O_2 and are preferentially lost after oxidant stress compared with more differentiated progeny (LPP-ECFC and EC clusters).

Given that HPP-ECFCs are highly proliferative with self-renewal capacity, we hypothesized that the preferential loss of HPP-ECFCs after H_2O_2 treatment would result in diminished proliferative capacity of EPC populations over a defined number of population doublings. To test this hypothesis, cord blood EPCs were treated with H_2O_2 and then allowed to grow to 90% confluence before cell monolayers were passaged. At each passage, viable ECs were enumerated, and CPDLs were calculated as previously described [22]. These studies revealed a marked reduction in CPDL of EPCs treated with 100 and 200 μM H_2O_2 compared with untreated controls (Fig. 2C). Together, these data suggest that H_2O_2 treatment of cord blood EPCs results in preferential loss of primitive HPP-ECFCs and an overall decline in proliferative capacity.

To determine whether the source of EPCs (cord blood vs. adult peripheral blood) affects oxidant responsiveness, adult peripheral blood-derived EPCs were established and treated with H_2O_2 in clonogenic progenitor assays. Similar to cord blood EPCs, adult EPCs were sensitive to H_2O_2 at multiple doses (Fig. 3A). Single-cell deposition assays also demonstrated an H_2O_2 dose-dependent reduction in total colonies formed (Fig. 3B). Consistent with previous data from our group [22], three of the adult EPC cell lines contained no HPP-ECFCs, whereas one adult EPC sample had nine colonies per 96 cells plated [22]. Upon H_2O_2 treatment, the number of HPP-ECFCs from this adult donor decreased to three at 50 μM H_2O_2, whereas no colonies were detected at the 100 and 200 μM H_2O_2 doses, consistent with data for cord blood EPCs showing that HPP-ECFCs are highly sensitive to oxidant stress. Interestingly, a marked reduction in the number of LPP-ECFCs was observed after 100 and 200 μM H_2O_2 treatment (Fig. 3C). Furthermore,

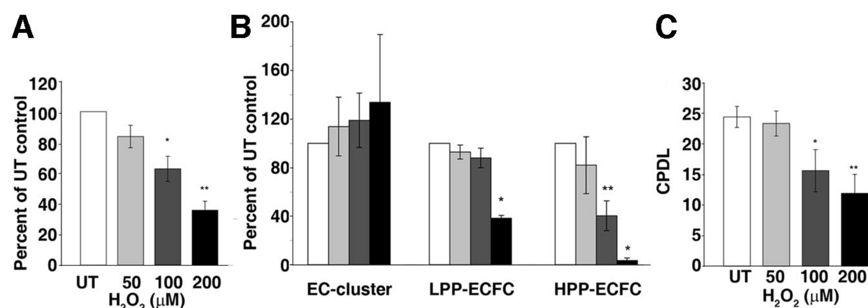

Figure 2. Clonogenic potential of single cord blood-derived endothelial progenitor cells (EPCs) and long-term replicative potential of cord blood-derived EPCs following H_2O_2 treatment. **(A):** Percentage of single cord blood-derived EPCs undergoing at least one cell division 14 days after H_2O_2 treatment. Results represent the mean ± SEM ($n = 3$; ∗, $p < .02$ and ∗∗, $p < .002$ by Student's paired *t* test). **(B):** Percentage of EC clusters, LPP-ECFCs, and HPP-ECFCs 14 days after H_2O_2 treatment. Results represent the mean ± SEM ($n = 4$). LPP-ECFC UT versus 200 μM and HPP-ECFC UT versus 200 μM, ∗, $p < .0001$; HPP-ECFC UT versus 100 μM, ∗∗, $p < .003$ by Student's paired *t* test. **(C):** Cumulative population doubling level of cord blood-derived EPCs on day 30 following H_2O_2 exposure. Results represent the mean ± SEM ($n = 4$; ∗, $p < .04$ and ∗∗, $p < .004$ by Student's unpaired *t* test). Abbreviations: CPDL, cumulative population doubling level; EC, endothelial cell; HPP-ECFC, high proliferative potential-endothelial colony-forming cell; LPP-ECFC, low proliferative potential-endothelial colony-forming cell; UT, untreated.

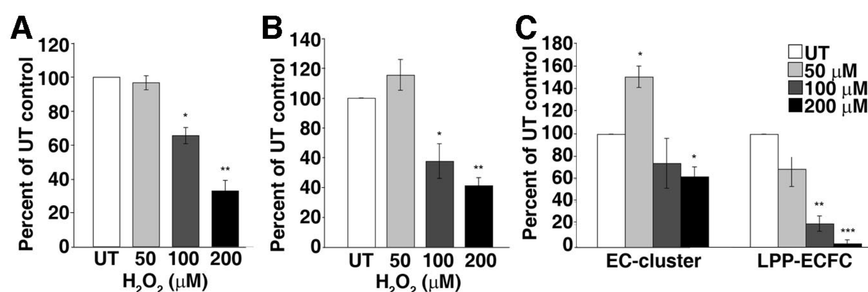

Figure 3. Clonogenic potential of adult blood-derived endothelial progenitor cells (EPCs) following H_2O_2 treatment. **(A):** Percentage of adult blood-derived EPCs forming colonies or clusters 7 days after H_2O_2 treatment. Results represent the mean ± SEM ($n = 4$; ∗, $p < .006$ and ∗∗, $p < .002$ by Student's paired *t* test). **(B):** Percentage of single adult blood-derived EPCs undergoing at least one division 14 days after H_2O_2 treatment. Results represent the mean ± SEM ($n = 4$; ∗, $p < .04$ and ∗∗, $p < .002$ by Student's paired *t* test). **(C):** Percentage of EC clusters and LPP-ECFCs 14 days after H_2O_2 treatment. Results represent the mean ± SEM ($n = 3$). EC cluster UT versus 50 μM and UT versus 200 μM, ∗, $p < .05$; LPP-ECFC UT versus 100 μM, ∗∗, $p < .008$; and LPP-ECFC UT versus 200 μM, ∗∗∗, $p < .002$ by Student's paired *t* test. Abbreviations: EC, endothelial cell; LPP-ECFC, low proliferative potential-endothelial colony-forming cell; UT, untreated.

the proportion of EC clusters was also decreased at the 200 μM H_2O_2 dose. These data are in contrast to cord blood EPC data, where LPP-ECFCs were only decreased at the 200 μM H_2O_2 dose, and no change was observed in EC clusters. Collectively, these data suggest that adult HPP-ECFCs, LPP-ECFCs, and EC clusters are more sensitive to oxidant stress than cord blood EPCs.

Enhanced H_2O_2-Induced Apoptosis of EPCs Is Dependent on the Catalytic Activity of ASK1

In performing clonogenic EPC assays, it appeared morphologically that EPCs were undergoing apoptosis in response to oxidant stress. This observation was intriguing since previous studies from our group directly link enhanced oxidant-induced apoptosis to reduced clonogenic capacity of hematopoietic progenitors [24, 25]. Based on this paradigm in hematopoietic progenitors, we next tested whether H_2O_2 induced apoptosis of cord blood EPCs using TUNEL assays. In multiple independent experiments, EPCs exhibited enhanced apoptosis after H_2O_2 treatment (Fig. 4A), consistent with clonogenic data. Similar results were obtained for EPCs derived from adult peripheral blood and HUVECs (data not shown), which is consistent with our observation that expression of endothelial nitric oxide synthase, manganese superoxide dismutase, glutathione peroxidase, and catalase do not differ appreciably between EPC cell sources (data not shown). To examine whether H_2O_2-induced apoptosis of EPCs was mediated through a redox signaling mechanism, EPCs were pretreated with NAC before treating with H_2O_2. These studies demonstrated that NAC pretreatment protected EPCs from H_2O_2-induced apoptosis to levels indistinguishable from untreated controls (Fig. 4A). Together these data suggest that H_2O_2-induced apoptosis contributes to the loss of EPCs via a redox-regulated apoptotic signaling mechanism.

Since H_2O_2-induced apoptosis in many cells types, including hematopoietic progenitors, is dependent on the redox-regulated protein ASK1 [28], we reasoned that EPCs may exhibit increased ASK1 activity after H_2O_2 treatment. To test this hypothesis, we evaluated H_2O_2-induced ASK1 activation in cord blood EPCs by Western blotting using a phospho-specific antibody that detects active ASK1. EPCs treated with 200 μM H_2O_2 had a significant increase in ASK1 activity compared with untreated controls (Fig. 4B shows one representative experiment of three with mean fold induction 2.1 ± 0.2; $p < .05$ for untreated vs. H_2O_2-treated). To determine whether the predisposition of EPCs to undergo H_2O_2-induced apoptosis was dependent on ASK1 kinase activity, EPCs were transduced with either a retroviral vector control encoding EGFP (MigR1) or a bicistronic retroviral vector encoding a catalytically inactive, dominant negative ASK1 cDNA [29] and EGFP (MigR1-ASK1-K709M) [25]. After transduction, EPCs were sorted by fluorescence cytometry for $EGFP^+$ cells and then evaluated for H_2O_2-induced apoptosis. Western analysis confirmed expression of the HA-tagged ASK1-K709M protein in transduced cells

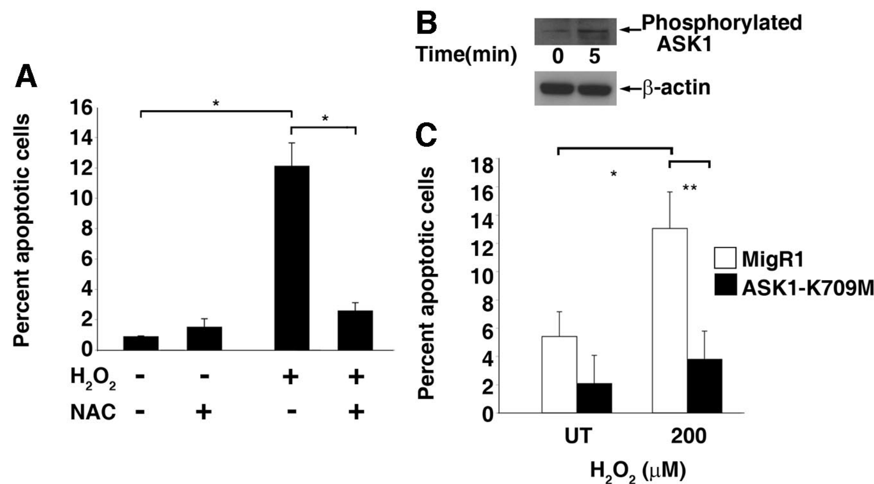

Figure 4. Enhanced ASK1-mediated apoptosis of cord blood-derived endothelial progenitor cells (EPCs) following H_2O_2 treatment. **(A):** Percentage of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-positive EPCs, with and without NAC pretreatment, 24 hours after H_2O_2 treatment. Data shown are the mean ± SEM of a representative experiment conducted in triplicate (n = 3 independent experiments with similar results; *, $p < .01$ by Student's paired t test). **(B):** Representative Western blot of phosphorylated ASK1 in EPCs treated with 200 μM H_2O_2. Similar results were seen in two other independent experiments. **(C):** Percentage of TUNEL-positive EPCs transduced with vector control (MigR1) or vector encoding ASK1-K709M 6 hours following no treatment (UT) or treatment with 200 μM H_2O_2. A representative experiment of three is shown. *, $p <$ .03 and **, $p < .005$ by Student's unpaired t test. Abbreviations: ASK1, apoptosis signal-regulating kinase 1; NAC, *N*-acetylcysteine; UT, untreated.

(data not shown). As expected, EPCs transduced with the vector control exhibited an increase in apoptosis after H_2O_2 treatment (Fig. 4C). In contrast, H_2O_2-treated EPCs transduced with MigR1-ASK1-K709M had levels of apoptosis that were comparable to untreated control EPCs (Fig. 4C). These data demonstrate an ASK1-dependent mechanism for the observed oxidant-induced apoptotic phenotype of EPCs.

Oxidant Stress Decreases Tube Formation via an ASK1-Dependent Mechanism

Resistance to oxidant-induced apoptosis would be a fundamental requirement for formation of new vessels at sites of ischemic injury. Given our observations outlined above, we tested whether oxidant stress diminished the tube-forming ability of cord blood EPCs using Matrigel assays as previously described [22]. Untreated or H_2O_2-treated EPCs were observed for initiation and maintenance of capillary-like structures over 5 days. At early time points (<6 hours), the initiation of capillary-like structures was similar between untreated and H_2O_2-treated EPCs (data not shown); however, by 24 hours, profound differences were observed (Fig. 5A). To examine whether aberrant tube formation in H_2O_2-treated EPCs was dependent on ASK1 kinase activity, Matrigel assays were conducted with transduced EPCs expressing ASK1-K709M. As expected, control transduced EPCs (MigR1) treated with H_2O_2 exhibited reduced total vessel length and closed network units (Fig. 5B, 5C). In contrast, EPCs expressing ASK1-K709M were completely protected from H_2O_2, as demonstrated by normalization of total vessel length and closed network units (Fig. 5B, 5C). These data implicate apoptosis, via enhanced ASK1 signaling, as a critical mechanism involved in diminished capillary tube-forming ability of EPCs after oxidant stress.

Reduced Vessel Formation of Oxidant-Treated EPCs In Vivo

To rigorously examine whether the functional capacity of EPCs was impaired after oxidant treatment, we transplanted EPCs into NOD/SCID mice using a well-established method to examine human-murine chimeric blood vessel formation in vivo [27]. Untreated EPCs formed chimeric vessels, which were perfused with mouse red blood cells (Fig. 6A, 6B). In contrast, grafts containing oxidant-treated EPCs formed few chimeric vessels. Collectively, these data show that oxidant-treated EPCs exhibit severely diminished functional capacity.

Discussion

Experimental data support a role for increased oxidant stress in the pathogenesis of vascular diseases [12–15]. Given that EC dysfunction is linked to vascular disease, it is imperative to understand how oxidative stress alters EC function. Furthermore, emerging data suggest that EPCs circulate in peripheral blood and reside in blood vessels for the initiation of vasculogenesis [22, 23]. However, understanding the effect of oxidative stress on EPC function has been hampered by the use of heterogeneous EPC populations contaminated with hematopoietic cells and the lack of clonogenic assays to test individual EPC function. Thus, we designed experiments to test whether oxidant stress alters EPC function using well-defined EC populations and clonogenic assays recently described by our group.

Our studies demonstrate that H_2O_2 treatment of both cord and adult blood-derived EPCs reduces their clonogenic capacity. Treatment of either established EPC cell lines or freshly isolated circulating MNCs, which contain EPC progenitors, with H_2O_2 resulted in a comparable reduction in EPC colony formation. These data indicate that both adherent and circulating EPCs are sensitive to oxidants. To examine whether primitive EPCs (HPP-ECFCs) versus more committed EPCs (LPP-ECFCs and EC clusters) were differentially sensitive to H_2O_2 treatment, single-cell deposition assays were conducted. Interestingly, cord blood HPP-ECFCs were more sensitive to H_2O_2 than LPP-ECFCs. These data are consistent with the decreased CPDLs in H_2O_2-treated EPCs, since the defining characteristics of HPP-ECFCs are their high proliferation rates and self-renewal capacity. Furthermore, Matrigel assays and xenograft transplant experiments, studies dependent on functional progenitor activity, demonstrate that oxidant treatment severely impairs the ability of EPCs to form blood vessels in vitro and in vivo.

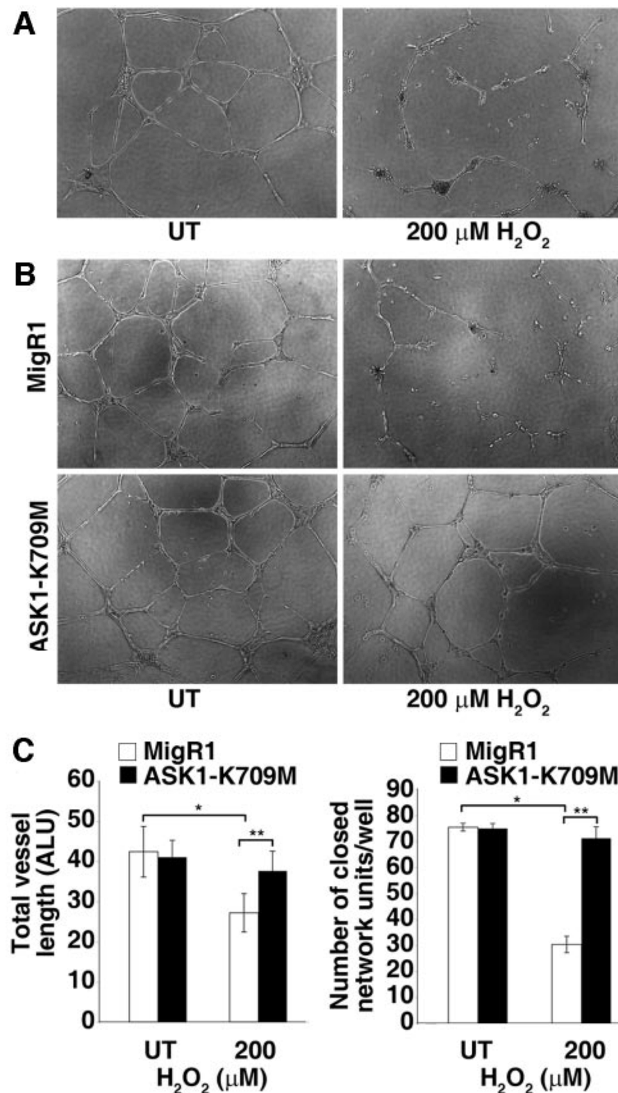

Figure 5. Tube-forming ability of cord blood-derived endothelial progenitor cells (EPCs) following H_2O_2 treatment. **(A):** Representative photomicrographs (magnification, ×10) of EPCs 24 hours following no treatment (UT) or treatment with 200 μM H_2O_2. **(B):** Representative photomicrographs (magnification, ×10) of EPCs transduced with vector control (MigR1) or vector encoding ASK1-K709M 24 hours following no treatment (UT) or treatment with 200 μM H_2O_2. Data shown are representative of three independent experiments. **(C):** Quantitation of capillary vessel length and vessel density 24 hours following 200 μM H_2O_2 treatment of EPCs transduced with either MigR1 or MigR1-ASK1-K709M. Data represent the average total capillary vessel length and average number of closed network units per well ± SEM ($n = 3$). Total vessel length: *, $p < .01$ and **, $p < .005$ by Student's paired t test. Closed network units: *, $p < .001$ and **, $p < .0001$ by Student's paired t test. Abbreviations: ALU, arbitrary length unit; ASK1, apoptosis signal-regulating kinase 1; UT, untreated.

An interesting finding of this study was the observation that adult-derived LPP-ECFCs were more sensitive to oxidant stress than cord blood-derived LPP-ECFCs. Single-cell deposition assays demonstrated a significant decrease in the colony-forming ability of adult-derived LPP-ECFCs at 100 and 200 μM H_2O_2 compared with cord blood-derived LPP-ECFCs. Furthermore, EC clusters from adult donors were also decreased at the highest H_2O_2 concentration tested, whereas there was no detectable change in cord blood EC cluster formation.

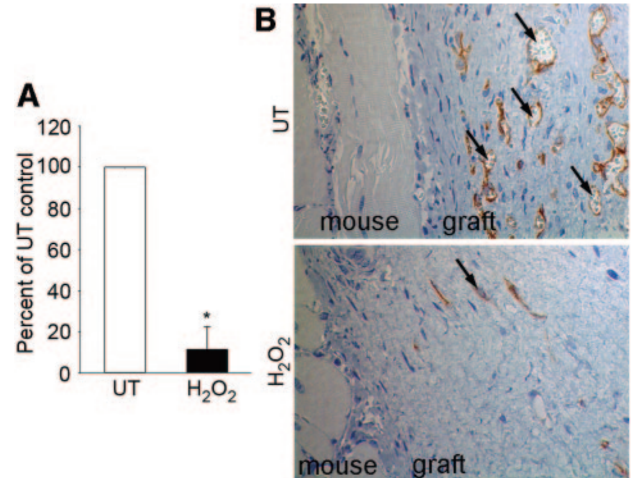

Figure 6. In vivo blood vessel formation of cord blood-derived endothelial progenitor cells (EPCs) following H_2O_2 treatment. **(A):** Percentage of human vessels perfused with murine red blood cells per square millimeter. EPCs were either UT or treated with 200 μM H_2O_2 prior to implantation into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Results represent the mean ± SEM ($n = 3$; *, $p < .04$ by Student's paired t test). **(B):** Photomicrographs (magnification, ×40) of xenograft implants and surrounding murine tissue 14 days after implantation into NOD/SCID mice. Grafts were stained with anti-human CD31 (brown) to identify human blood vessels, and arrows indicate representative human vessels perfused with murine red blood cells. Abbreviation: UT, untreated.

These data are intriguing and suggest the possibility that a loss of circulating EPCs through aging may predispose to endothelial dysfunction and vascular disease. A similar mechanism has been proposed for aging of the hematopoietic system, where hematopoietic stem cells (HSCs) decrease in number and exhibit reduced self-renewal, homing, and engrafting ability, which result in a diminished capacity of aged HSCs to respond to hematopoietic stress [30, 31]. Whether oxidant damage directly leads to an age-related decline in EPC function is an important unanswered question, which we are currently pursuing based on our observations. However, compelling evidence suggest that numerous disease states enhance oxidant stress in vivo prior to clinically significant vascular disease, including homocystinemia, diabetes, hypercholesterolemia, and tobacco use [12, 32, 33], supporting the notion that increased endogenous oxidant stress promotes vascular pathology. Furthermore, an age-related decline in EPC function may explain in part the limited and variable success of clinical trials testing the efficacy of antioxidants in the treatment of patients with vascular disease.

Our data showing enhanced EPC sensitivity to oxidative stress are in contrast to previous studies [20, 21]. Dernbach et al. reported that EPCs are resistant to oxidant stress and uniquely equipped to repair damaged vessels in the hyperoxic environment observed in ischemic tissues [20]. However, the circulating EPCs used by Dernbach et al. [20] co-express hematopoietic macrophage/monocyte and endothelial antigens and are not capable of self-renewal divisions. Thus, these cells have been characterized as circulating angiogenic cells [34, 35] rather than clonogenic EPCs. In contrast, EPCs isolated by our methods and those of others express only endothelial antigens and display clonogenic and self-renewal capacity, which are hallmarks of stem and progenitor cells [22]. Therefore, the discrepancy between these studies is likely because of differences in cell types being analyzed.

On the other hand, He et al. used techniques similar to those in our study to isolate adult EPCs and showed that HUVECs and

coronary artery endothelial cells (CAECs) are more sensitive to oxidant stress than adult peripheral blood-derived EPCs [21]. Thus, He et al. [21] concluded that adult EPCs may be resistant to oxidants generated in an ischemic environment. However, recent observations made by our group lend further insight and interpretation of these data (unpublished data). For example, HUVECs and CAECs were used as mature endothelial cell line controls by He et al. [21]. However, vessel wall-derived ECs, including HUVECs and CAECs, contain a complete hierarchy of clonogenic EPCs and are highly enriched for HPP-ECFCs [23], making HUVECs and CAECs more immature EPC cell lines compared with adult circulating peripheral blood EPCs that contain few HPP-ECFCs. In our hands, EPCs derived from either HUVECs, cord blood, or adult peripheral blood underwent similar levels of H_2O_2-induced apoptosis. However, single-cell deposition assays demonstrated increased sensitivity of adult EPCs compared with cord blood EPCs. Collectively, these findings emphasize the need to carefully define the cell types being analyzed and the power of using clonogenic assays to demonstrate functional differences in EPC populations.

Although it has been hypothesized that EPCs resist oxidant-induced apoptosis at sites of ischemia, our data clearly show that EPCs are sensitive to H_2O_2-induced apoptosis via the redox-dependent ASK1 pathway. Furthermore, the tube-forming ability of cord blood EPCs is also diminished after oxidant stress via ASK1 activation. ASK1 is a mitogen-activated protein kinase that is negatively regulated by multiple redox-sensitive binding partners, including thioredoxin, glutathione *S*-transferases, and glutaredoxin [29, 36–42]. Although the contribution of individual regulatory proteins in controlling ASK1 activity is unknown, it is clear that oxidation of these redox-sensitive proteins results in ASK1 activation, which is capable of initiating apoptosis via the downstream effectors p38 and c-jun N-terminal kinase [29]. Interestingly, previous studies in HUVECs, which contain a high frequency of HPP-ECFCs, demonstrate an important role for thioredoxin in protecting ECs from oxidant-induced apoptosis. Haendeler et al. showed that statins induce *S*-nitrosylation of thioredoxin, resulting in increased thioredoxin-reactive oxygen radical scavenging activity and improved survival [43]. In a separate report, H_2O_2 treatment resulted in thioredoxin degradation, thereby decreasing reactive oxygen radical scavenging ability and increasing apoptosis [44]. Together, these data are consistent with our findings that H_2O_2-induced apoptosis in EPCs is ASK1-dependent, since ASK1 activity is directly linked to available thioredoxin (reduced state).

Summary

In summary, our data demonstrate that both clonogenic cord and adult blood-derived EPCs are sensitive to oxidant stress. Furthermore, EPCs treated with oxidants undergo increased apoptosis and decreased tube formation via ASK1 activation. Collectively, these studies highlight the complexity of using autologous or cord blood-derived EPCs for vascular repair in ischemic injury and emphasize the importance of designing therapeutic strategies to protect EPCs against oxidant stress to enhance new vessel formation.

Disclosures

D.A.I. and M.C.Y. own stock in EndGenitor Technologies, Inc. (Indianapolis, IN). They also have acted as consultants to and served as officers or members of the Board of EndGenitor Technologies, Inc., within the last 2 years.

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References

1 Urbich C, Dimmeler S. Endothelial progenitor cells: Characterization and role in vascular biology. Circ Res 2004;95:343–353.
2 Rafii S, Lyden D. Therapeutic stem and progenitor cell transplantation for organ vascularization and regeneration. Nat Med 2003;9:702–712.
3 Khakoo AY, Finkel T. Endothelial progenitor cells. Annu Rev Med 2005;56:79–101.
4 Schatteman GC. Adult bone marrow-derived hemangioblasts, endothelial cell progenitors, and EPCs. Curr Top Dev Biol 2004;64:141–180.
5 Iwami Y, Masuda H, Asahara T. Endothelial progenitor cells: Past, state of the art, and future. J Cell Mol Med 2004;8:488–497.
6 Hristov M, Weber C. Endothelial progenitor cells: Characterization, pathophysiology, and possible clinical relevance. J Cell Mol Med 2004; 8:498–508.
7 Murasawa S, Asahara T. Endothelial progenitor cells for vasculogenesis [Review]. Physiology (Bethesda) 2005;20:36–42.
8 Ingram DA, Caplice NM, Yoder MC. Unresolved questions, changing definitions, and novel paradigms for defining endothelial progenitor cells. Blood 2005;106:1525–1531.
9 Gothert JR, Gustin SE, van Eekelen JA et al. Genetically tagging endothelial cells in vivo: Bone marrow-derived cells do not contribute to tumor endothelium. Blood 2004;104:1769–1777.
10 Stadtfeld M, Graf T. Assessing the role of hematopoietic plasticity for endothelial and hepatocyte development by non-invasive lineage tracing. Development 2005;132:203–213.
11 Peters BA, Diaz LA, Polyak K et al. Contribution of bone marrow-derived endothelial cells to human tumor vasculature. Nat Med 2005;11: 261–262.
12 Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: Molecular and cellular mechanisms. Hypertension 2003;42: 1075–1081.
13 Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: The role of oxidant stress. Circ Res 2000;87:840–844.
14 Touyz RM. Reactive oxygen species, vascular oxidative stress, and redox signaling in hypertension: What is the clinical significance? Hypertension 2004;44:248–252.
15 Ogita H, Liao J. Endothelial function and oxidative stress. Endothelium 2004;11:123–132.
16 Gulati R, Jevremovic D, Peterson TE et al. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. Circ Res 2003;93:1023–1025.
17 Schmeisser A, Garlichs CD, Zhang H et al. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. Cardiovasc Res 2001;49: 671–680.
18 Fernandez Pujol B, Lucibello FC, Gehling UM et al. Endothelial-like cells derived from human CD14 positive monocytes. Differentiation 2000;65:287–300.
19 Rohde E, Malischnik C, Thaler D et al. Blood monocytes mimic endothelial progenitor cells. Stem Cells 2006;24:357–367.
20 Dernbach E, Urbich C, Brandes RP et al. Antioxidative stress-associated genes in circulating progenitor cells: Evidence for enhanced resistance against oxidative stress. Blood 2004;104:3591–3597.
21 He T, Peterson TE, Holmuhamedov EL et al. Human endothelial progenitor cells tolerate oxidative stress due to intrinsically high expression of manganese superoxide dismutase. Arterioscler Thromb Vasc Biol 2004;24:2021–2027.
22 Ingram DA, Mead LE, Tanaka H et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. Blood 2004;104:2752–2760.
23 Ingram DA, Mead LE, Moore DB et al. Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. Blood 2005;105: 2783–2786.

24 Saadatzadeh MR, Bijangi-Vishehsaraei K, Hong P et al. Oxidant hypersensitivity of Fanconi anemia type C-deficient cells is dependent on a redox-regulated apoptotic pathway. J Biol Chem 2004;279: 16805–16812.
25 Bijangi-Vishehsaraei K, Saadatzadeh MR, Werne A et al. Enhanced TNF-alpha-induced apoptosis in Fanconi anemia type C-deficient cells is dependent on apoptosis signal-regulating kinase 1. Blood 2005;106: 4124–4130.
26 Haneline LS, Li X, Ciccone SL et al. Retroviral-mediated expression of recombinant Fancc enhances the repopulating ability of *Fancc−/−* hematopoietic stem cells and decreases the risk of clonal evolution. Blood 2003;101:1299–1307.
27 Schechner JS, Nath AK, Zheng L et al. In vivo formation of complex microvessels lined by human endothelial cells in an immunodeficient mouse. Proc Natl Acad Sci U S A 2000;97:9191–9196.
28 Matsukawa J, Matsuzawa A, Takeda K et al. The ASK1-MAP kinase cascades in mammalian stress response. J Biochem (Tokyo) 2004;136: 261–265.
29 Ichijo H, Nishida E, Irie K et al. Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. Science 1997;275:90–94.
30 Geiger H, Van Zant G. The aging of lympho-hematopoietic stem cells. Nat Immunol 2002;3:329–333.
31 Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood 2005;106:1479–1487.
32 Davi G, Falco A. Oxidant stress, inflammation and atherogenesis. Lupus 2005;14:760–764.
33 Loomans CJ, De Koning EJ, Staal FJ et al. Endothelial progenitor cell dysfunction in type 1 diabetes: Another consequence of oxidative stress? Antioxid Redox Signal 2005;7:1468–1475.
34. Yoder MC, Mead LE, Prater D et al. Re-defining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. Blood 2006 [Epub ahead of print].
35 Rehman J, Li J, Orschell CM et al. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. Circulation 2003;107:1164–1169.
36 Saitoh M, Nishitoh H, Fujii M et al. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. EMBO J 1998; 17:2596–2606.
37 Cho SG, Lee YH, Park HS et al. Glutathione S-transferase mu modulates the stress-activated signals by suppressing apoptosis signal-regulating kinase 1. J Biol Chem 2001;276:12749–12755.
38 Gilot D, Loyer P, Corlu A et al. Liver protection from apoptosis requires both blockage of initiator caspase activities and inhibition of ASK1/JNK pathway via glutathione S-transferase regulation. J Biol Chem 2002;277: 49220–49229.
39 Liu H, Nishitoh H, Ichijo H et al. Activation of apoptosis signal-regulating kinase 1 (ASK1) by tumor necrosis factor receptor-associated factor 2 requires prior dissociation of the ASK1 inhibitor thioredoxin. Mol Cell Biol 2000;20:2198–2208.
40 Song JJ, Rhee JG, Suntharalingam M et al. Role of glutaredoxin in metabolic oxidative stress. Glutaredoxin as a sensor of oxidative stress mediated by H2O2. J Biol Chem 2002;277:46566–46575.
41 Song JJ, Lee YJ. Differential role of glutaredoxin and thioredoxin in metabolic oxidative stress-induced activation of apoptosis signal-regulating kinase 1. Biochem J 2003;373:845–853.
42 Dorion S, Lambert H, Landry J. Activation of the p38 signaling pathway by heat shock involves the dissociation of glutathione S-transferase Mu from Ask1. J Biol Chem 2002;277:30792–30797.
43 Haendeler J, Hoffmann J, Zeiher AM et al. Antioxidant effects of statins via S-nitrosylation and activation of thioredoxin in endothelial cells: A novel vasculoprotective function of statins. Circulation 2004;110:856–861.
44 Haendeler J, Popp R, Goy C et al. Cathepsin D and H2O2 stimulate degradation of thioredoxin-1: Implication for endothelial cell apoptosis. J Biol Chem 2005;280:42945–42951.