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Paracrine factors secreted by endothelial progenitor cells prevent oxidative stress-induced apoptosis of mature endothelial cells

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ABSTRACT

Endothelial progenitor cells (EPC) play a fundamental role in tissue regeneration and vascular repair. Current research suggests that EPC are more resistant to oxidative stress as compared to differentiated endothelial cells. Here we hypothesized that EPC not only possess the ability to protect themselves against oxidative stress but also confer this protection upon differentiated endothelial cells by release of paracrine factors. To test this hypothesis, HUVEC incubated with conditioned medium obtained from early EPC cultures (EPC-CM) were exposed to H_2O_2 to assess the accumulation of intracellular ROS, extent of apoptosis and endothelial cell functionality. Under oxidative stress conditions HUVEC treated with EPC-CM exhibited substantially lower levels of intracellular oxidative stress (0.2 ± 0.02 vs. 0.4 ± 0.03 relative fluorescence units, $p < 0.05$) compared to control medium. Moreover, the incubation with EPC-CM elevated the expression level of antioxidant enzymes in HUVEC (catalase: 2.6 ± 0.4; copper/zinc superoxide dismutase (Cu/ZnSOD): 1.6 ± 0.1; manganese superoxide dismutase (MnSOD): 1.4 ± 0.1-fold increase compared to control, all $p < 0.05$). Furthermore, EPC-CM had the distinct potential to reverse the functional impairment of HUVEC as measured by their capability to form tubular structures in vitro. Finally, incubation of HUVEC with EPC-CM resulted in a significant reduction of apoptosis (0.34 ± 0.01 vs. 1.52 ± 0.12 relative fluorescence units, $p < 0.01$) accompanied by an increased expression ratio of the anti/pro-apoptotic factors Bcl-2/Bax to 2.9 ± 0.7-fold (compared to control, $p < 0.05$). Most importantly, neutralization of selected cytokines such as VEGF, HGF, IL-8 and MMP-9 did not significantly reverse the cyto-protective effect of EPC-CM ($p > 0.05$), suggesting that soluble factors secreted by EPC, possibly via broad synergistic actions, exert strong cyto-protective properties on differentiated endothelium through modulation of intracellular antioxidant defensive mechanisms and pro-survival signals.

1. Introduction

The entire cardiovascular system critically depends on a structurally and functionally intact vascular endothelium. The loss of endothelial integrity and function is known to play a pivotal role in the initiation and progression of vascular dysfunction occurring secondary to diseases such as hypertension or diabetes mellitus ultimately leading to complications including myocardial infarction or stroke [1]. A major initiator of endothelial injury is oxidative stress which results from an imbalanced state of increased reactive oxygen species (ROS) generation and insufficient intracellular antioxidants [2].

As a matter of fact both, oxidative stress due to excessive ROS formation and a defective capacity to detoxify intracellular oxidants are common features of several cardiovascular diseases although the cause–effect relationship between oxidative damage and cardiovascular dysfunction is not completely understood [3].

Growing evidence suggests that EPC contribute to the structural integrity of the vasculature by promoting angiogenesis through the secretion of angiogenic growth factors as well as by their replicative potential and ability to differentiate into mature vascular endothelial cells [4,5]. Previous studies have reported that EPC are

Abbreviations: EPC, Endothelial progenitor cells; HUVEC, Human umbilical vein endothelial cells; EPC-CM, EPC-conditioned medium; HUVEC-CM, HUVEC-conditioned medium; PBMC, Peripheral blood mononuclear cells; DHE, Dihydroethidine; ROS, Reactive oxygen species; PEG-SOD, Polyethylene glycol-superoxide dismutase; H_2O_2, Hydrogen peroxide; O_2^-, Superoxide anion; Cu/ZnSOD, Copper/zinc superoxide dismutase; MnSOD, Manganese superoxide dismutase.

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more resistant to oxidative stress than differentiated endothelial cells due to an elevated expression of the intracellular antioxidant enzymes catalase, glutathione peroxidase and manganese superoxide dismutases (MnSOD) [2]. This resistance against oxidative injury likely allows EPC to survive and exert their angiogenic and vascular repair functions in microenvironments with elevated ROS levels like ischemic or inflamed tissue.

In light of the regenerative properties of EPC we hypothesized that EPC, in particular the subpopulation termed early EPC, may protect not only themselves but also resident endothelial cells from the detrimental effect of oxidative stress through paracrine mechanisms. In the present study we sought to determine, whether soluble factors secreted by EPC support resistance of endothelial cells against ROS toxicity thereby preserving their viability and functional activity under conditions of oxidative stress.

2. Materials and methods

2.1. Cell isolation and culture

HUVEC were isolated from umbilical cord by collagenase digestion [6] and cultivated in complete endothelial cell growth medium (EGM-2-MV, Lonza, Switzerland) containing 5% fetal bovine serum (FBS). All experiments were performed using cells between passages 2 and 6. To harvest endothelial progenitor cells (EPC), peripheral blood mononuclear cells (PBMC) were isolated from blood of healthy human volunteers by density gradient centrifugation with Histopaque®-1077 (Sigma–Aldrich, Switzerland) as described previously [7]. PBMC were plated on culture dishes coated with human fibronectin (Clonetech, Switzerland) and maintained in EGM-2-MV containing 5% FBS. After 4 days in culture, non-adherent cells were removed by a single washing step with phosphate-buffered saline (PBS). Adherent cells were trypsinized, passaged and maintained in culture till day 7. EPC were characterized by uptake of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine-labeled acetylated low density lipoprotein (Dil-Ac-LDL, Harbor Bio-products) and BS-1 lectin (Sigma) staining, as well as flow cytometry analysis of the following surface markers: CD34, CD133, CD45, CD14, KDR, CD31, VE-cadherin (CD144) and MCAM (CD146) as published previously [8,9].

2.2. Preparation of conditioned medium

To produce human EPC and HUVEC conditioned medium (EPC-CM and HUVEC-CM, respectively), EPC and HUVEC were cultured for 72 h under hypoxic conditions (1.5% O_2, 5% CO_2, 93.5% N_2) using a humidified gas-sorted anoxic incubator-gloved box (InVivo2 400, Ruskin, UK). A growth factor-free endothelial cell basal medium-2 (EBM-2, Lonza, Switzerland) with 1% FBS was employed in this step and served as control medium throughout the experiments. After incubation, the culture supernatant was centrifuged, sterile filtered with a 0.22 μm filter (TPP, Switzerland) and stored at −80 °C until use.

2.3. Assessment of intracellular ROS

2×10^4 HUVEC were plated per well in an 8-well culture slides (BD, Switzerland) in EGM-2-MV +5% FBS. After overnight starvation, the monolayers were incubated with EPC-CM or control medium and treated with 500 μM H_2O_2 for 8 h. In addition a set of wells were conditioned with 100 U/ml of the superoxide scavenger PEG-SOD (Sigma–Aldrich, Switzerland). To measure the accumulation of intracellular superoxide HUVEC were loaded with 5 μM DHE (Invitrogen, Switzerland) 30 min before the experiment termination. After washing the cells once with PBS, cells were counterstained with DAPI (Invitrogen, Switzerland). Images were acquired with a fluorescent microscope (Nikon Eclipse 800, Japan) at 200-fold magnification and the fluorescence intensity was assessed by Adobe Photoshop CS4. Fluorescent intensity was expressed as the ratio of the DHE fluorescence (red) to the cell number.

2.4. Immunoblot analysis

After exposure to 500 μM H_2O_2 for 24 h HUVEC were lysed in RIPA buffer containing the Protease Inhibitor Cocktail V (Calbiochem, Switzerland). Cell lysates were centrifuged at 4 °C for 20 min and the supernatant was harvested for further analysis. Equal amount of sample proteins were resolved in 12% acrylamide gels as previously described [10], blotted on nitrocellulose and marked using the following primary antibodies: rabbit anti-catalase (219010, Calbiochem), sheep anti-Cu/ZnSOD (574597, Calbiochem), sheep anti-MnSOD (574596, Calbiochem), rabbit anti-Bcl-2 (13-8800, Invitrogen), rabbit anti-Bax (sc-6236, Santa Cruz) and mouse anti-actin (MAB1501, Chemicon) as control.

2.5. In vitro capillary formation

2×10^4 HUVEC/well were plated in Matrigel™-coated 24-well plates (BD, Switzerland). Following 8 h of incubation with EPC-CM or control medium, digital images of the forming capillary-like structures were acquired. In vitro angiogenic activity was assessed measuring the total length and the number of sprouts per high power field with the aid of ImageJ. Calculations were performed in three random high power fields (HPF) and in three independent experiments, respectively.

2.6. Cytokine array

In order to screen the soluble factors contained in EPC-CM, a commercially available antibody array (AAH-CYT-2000, Ray Biotech, USA) for the detection of 174 human growth factors and cytokines was used. HUVEC-CM and the basal medium containing 1% FCS (control medium) served as a reference. The chemiluminescent signal of each factor on the array was acquired by ChemiDoc™ XRS (Bio-Rad AG, Switzerland) and the intensity measured by ImageJ. After background subtraction, the level of the cytokines in EPC-CM was expressed in a semi-quantitative fashion and as fold-increase over HUVEC-CM and basal medium.

2.7. Assessment of apoptotic cell death

5×10^3 HUVEC were plated per well of a 96-well plate. Before the experiment HUVEC were starved overnight in control medium. Thereafter, cells were simultaneously incubated with EPC-CM or control medium and 500 μM H_2O_2 for 18 h.

Induction of apoptosis was assessed by measuring the caspase-3 and caspase-7 activity using the Apo-ONE® homogenous caspase-3/7 kit (Promega, Switzerland). Fluorescence units were measured by the Tecan Safire reader (Tecan, Austria) and values were expressed as percentage relative to the values obtained from control group. To assure that the cyto-protective effect of EPC-CM is specific and dose-dependent, the level of apoptosis was measured in cells incubated with HUVEC-CM and serial dilutions of EPC-CM in control medium to reach a final concentration of 0, 25, 50 and 100% EPC-CM.

2.8. Selected cytokine neutralization

In order to explore whether single soluble factors are responsible for exerting the cyto-protective/anti-apoptotic effect of