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Overexpression of hypoxia-inducible factor-1 alpha improves vasculogenesis-related functions of endothelial progenitor cells

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ABSTRACT

Postnatal vasculogenesis is mediated by mobilization of endothelial progenitor cells (EPCs) from bone marrow and homing to ischemic tissues. This feature emphasizes this cell type for cell-based therapies aiming at the improvement of neovascularization in tissue engineering applications and regenerative medicine. In animal models, it was demonstrated that implantation of EPCs from cord blood (cbEPCs) led to the formation of a complex functional neovasculature, whereas EPCs isolated from adult peripheral blood (pbEPCs) showed a limited vasculogenic potential, which may be attributed to age-related dysfunction. Recently, it was demonstrated that activation of hypoxia-inducible factor-1α (Hif-1α) improves cell functions of progenitor cells of mesenchymal and endothelial origin. Thus, we hypothesized that overexpression of Hif-1α may improve the vasculogenesis-related phenotype of pbEPCs. In the present study, we overexpressed Hif-1α in pbEPCs and cbEPCs by using recombinant adenoviruses and investigated effects on stem cell- and vasculogenesis-related cell parameters. Overexpression of Hif-1α enhanced proliferation, invasion, cell survival and in vitro capillary sprout formation of both EPC populations. Migration was increased in cbEPCs upon Hif-1α overexpression, but not in pbEPCs. Cellular senescence was decreased in pbEPCs, while remained in cbEPCs, which showed, as expected, intrinsically a dramatically lower senescent phenotype in relation to pbEPCs. Similarly, the colony-formation capacity was much higher in cbEPCs in comparison to pbEPCs and was further increased by Hif-1α overexpression, whereas Hif-1α transduction exerted no significant influence on colony formation of pbEPCs. In summary, our experiments illustrated multifarious effects of Hif-1α overexpression on stem cell and vasculogenic parameters. Therefore, Hif-1α overexpression may represent a therapeutic option to improve cellular functions of adult as well as postnatal EPCs.

1. Introduction

Endothelial progenitor cells (EPCs) play a decisive role in postnatal vasculogenesis. They are located in the bone marrow from which they can be mobilized into the blood stream through the action of factors such as VEGF (Asahara et al., 1997), angiopoietin 1 (Hattori et al., 2001), stromal-derived factor 1 (Hattori et al., 2001) and statins (Llevadot et al., 2001) circulating EPCs home to sites of new blood vessel formation where they support neovascularization by incorporation into damaged blood vessels and by the induction of angiogenesis via secretion of pro-angiogenic factors. Therefore, EPCs are directly involved in many regeneration processes where neovascularization is required.

Because of their important physiological role in neovascularization, EPCs represent a very interesting cell source for applications in the field of regenerative medicine and tissue engineering. In this context it was shown that these cells can regenerate infarcted myocardium by supporting neovascularization (Orlic et al., 2001; Kawamoto et al., 2001). The fact that EPCs can be easily isolated from peripheral blood, thereby preventing donor site morbidity, highlight this cell type for tissue engineering applications which are in principle based on the use of autologous cells. Moreover, ex vivo vascularization of tissue engineered grafts by using this cell line would represent a promising strategy to improve graft survival after transplantation.

EPCs, also referred to as outgrowth endothelial cells (OEC) (Yoder, 2012; Yoder et al., 2007) can be isolated from peripheral blood as well as from cord blood. We (Finkenzeller et al., 2009), as well as others (Au et al., 2008; Melero-Martin et al., 2007) have illustrated that EPCs isolated from adult peripheral blood (pbEPCs) were unable to form blood vessels upon implantation, whereas EPCs isolated from cord blood (cbEPCs) showed a high vasculogenic potential. The reason for

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this functional difference is still unclear, but it may be assumed that the impaired vasculogenic potential of adult pbEPCs may be attributed to age-related dysfunction (Shelley et al., 2012).

A promising strategy to promote the vasculogenic potential of adult EPCs could consist of overexpression of the proangiogenic master transcription factor hypoxia-inducible factor-1 (Hif-1) in EPCs. Hif-1 is composed of two subunits, the oxygen-dependent subunit Hif-1α and the constitutively expressed Hif-1β which heterodimerize and bind to the so called hypoxia response element in promoters of several genes that influence angiogenesis, erythropoiesis and cell metabolism in response to tissue hypoxia (Wang and Semenza, 1995; Wang et al., 1995; Jiang et al., 1996; Huang et al., 2010). Under normoxic conditions, prolyl hydoxylase is activated and hydroxylates proline residues 402 and 564 of Hif-1α thereby linking Hif-1α to the von Hippel–Lindau protein (pVHL), an E3 ubiquitin ligase. This leads to degradation of Hif-1α in the proteosome (Kallio et al., 1999; Ivan et al., 2001; Maxwell et al., 1999). Under hypoxic conditions, Hif-1α hydroxylation is inhibited and Hif-1α accumulates in the nucleus where it forms a heterodimer with the Hif-1β subunit and activates several Hif-responsive proangiogenic genes such as VEGF (Kallio et al., 1998).

Hif-1 was shown to improve cell functions of progenitor cells. In this context, it was demonstrated that activation of Hif-1α in mesenchymal stem cells (MSC) is associated with increased proliferation (Lampert et al., 2015; Peng et al., 2015), migration (Lampert et al., 2015; Okuyama et al., 2006) and decreased senescence (Tsai et al., 2011). Similarly, it was recently shown that hypoxia inhibits cellular senescence of EPCs via the Hif-1α-TWIST-p21 pathway (Lee et al., 2013). Therefore, a gene therapeutic approach to overexpress Hif-1α in EPCs could represent an attractive therapeutic option to improve the vasculogenic potential of peripheral blood-derived adult EPCs, thereby making this cell type available as an autologous cell source for vascularization of implants in tissue engineering applications.

In the present study, we have overexpressed Hif-1α in adult pbEPCs and postnatal cbEPCs by using recombinant adenoviruses. Effects of Hif-1α overexpression on functional stem cell parameters such as senescence and colony formation were investigated as well as effects on vasculogenesis-related in vitro parameters such as proliferation, migration, invasion, cell survival and in vitro capillary formation in two- and three-dimensional in vitro angiogenesis assays.

2. Materials and methods

2.1. Cell culture

Human peripheral blood EPCs (pbEPCs) and human cord blood EPCs (cbEPCs) were isolated and expanded as previously described (Finkenzeller et al., 2007). In brief, mononuclear cells (MNCs) were isolated from 50 ml of peripheral blood or cord blood by density gradient centrifugation with biocoll separation solution (Biochrom, Berlin, Germany). Cells were plated on 25 cm^2 culture flasks coated with rat type I collagen (50 μg/ml) in EGM-2 (Lonza, Cologne, Germany) supplemented with single quots (hEGF, VEGF, hFGF-B, R3-IGF-1, ascorbic acid, heparin, gentamicin/amphotericin-B) and 10% FCS and cultivated at 37 °C, 5% CO_2 in a humidified atmosphere. After 4 days in culture, non-adherent cells were removed by washing with phosphate buffered saline (PBS), new media was applied, and the culture was maintained for another 4 to 5 weeks. Media were changed every 3 days. After reaching about 80% confluence, cells were trypsinized and seeded into 75 cm^2 culture flasks for expansion. EPCs from passage 2–5 were used for experiments. In order to confirm the endothelial phenotype, EPCs were analyzed for cell surface expression of endothelial markers. EPCs were positive for CD31, vWF, VEGFR-2, VE-Cadherin, CD105 and CD146, whereas the hematopoietic cell surface markers CD45 and CD14 were not expressed. Moreover, EPCs showed a cobblestone morphology and were able to incorporate dil-labeled acetylated low density lipoprotein. Usage of the cells was approved by the ethics committee of the University of Freiburg.

2.2. Adenoviral infection

pbEPCs and cbEPCs were infected with commercially available Hif-1α or GFP control adenovirus (Cell Biolabs, San Diego, USA) at a multiplicity of infection (MOI) of 1. Both adenoviruses contain a green fluorescent protein (GFP) open reading frame as a marker gene under control of a constitutive active CMV promoter.

2.3. Hif-1α ELISA

cbEPCs, as well as pbEPCs were seeded in triplicates in 6-well plates at a density of 2×10^5 cells per well. The next day, when both cell lines became adherent, they were transduced with Hif-1α or GFP control adenovirus at a MOI of 1. Cells were incubated for 48 h subsequently. Afterwards, cell lysates were prepared and by using the BCA protein assay kit (Thermo Scientific, Rockford, USA) total protein was quantified. The evaluation of Hif-1α protein in cell lysates was realized with the Hif-1α human ELISA Kit (Abcam, Cambridge, UK) according to manufacturer's instructions. Hif-1α protein content was normalized to total protein content and expressed as ng Hif-1α per mg total protein.

2.4. Cell proliferation assay

Both pbEPCs and cbEPCs were infected with Hif-1α or GFP control adenovirus at MOI = 1 and incubated for 48 h. After this, cells were trypsinized and seeded in triplicates into 48-well plates at a density of 4×10^3 cells per well in ECGM, 50 μg/ml gentamicin, 10% FCS. Cells were then incubated for the indicated time periods. Medium was changed every third day. Cell numbers were determined via CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Heidelberg, Germany) according to manufacturer's instructions. Microtiter plates were analyzed at 490 nm using a microtiter plate reader.

2.5. Apoptosis assay

Apoptosis was assessed by quantification of fragmented DNA by ELISA (Cell Death Detection ELISA kit, Roche Diagnostics, Germany). This assay allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of apoptotic cells. cbEPCs as well as pbEPCs were infected with Hif-1α or GFP control adenovirus at MOI = 1 and incubated for 2 days. Thereafter, cells were detached and seeded in triplicates into 12-well plates at a density of 5×10^4 cells per well. Following attachment, cells were imbedded in ECGM with 50 μg/ml gentamicin containing either 10% or 0% FCS. After further 48 h, cells were directly lysed in incubation buffer and oligonucleosomes were extracted from the cytoplasmic fraction of the cells by incubation at room temperature for 30 min with vigorous shaking. The extracts were centrifuged for 10 min at 12000 rpm at room temperature. The resulting supernatants were incubated with peroxidase-labeled anti-DNA antibody and biotinylated anti-histone antibody in streptavidin-coated microtiter plates following manufacturer's instructions. After washing, peroxidase substrate ABTS (2,2′-Azino-di[3-ethylbenzthiazolin-sulfonat]) was added to develop and visualize binding of mono- and oligonucleosomal DNA. Microtiterplates were analyzed at 410 nm using a microtiter plate reader.

2.6. Migration and invasion assay

pbEPCs and cbEPCs were transduced by Hif-1α or GFP control adenovirus at MOI = 1 and incubated for 48 h. Cell migration and invasion was estimated in 12-well transwell cell culture chambers by means of a transparent membrane with pores of 8 μm (BD Biosciences, Heidelberg, Germany). Regarding invasion assays the membrane was coated additionally overnight at room temperature with 100 μl of growth factor