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Apolipoprotein A-I enhances proliferation of human endothelial progenitor cells and promotes angiogenesis through the cell surface ATP synthase

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ABSTRACT

Background: Human endothelial progenitor cells (hEPC) correspond to a subtype of stem cells which, in the presence of angiogenic stimuli, can be mobilized from bone marrow to circulation and then recruited to the damaged endothelium, where they differentiate into mature endothelial cells. High-density lipoproteins (HDL) increase the level and functionality (proliferation, migration, differentiation, angiogenesis capacity) of circulating hEPC; however, the contribution of receptors for HDL and/or apolipoprotein A-I (apoA-I), the main HDL apolipoprotein, in these effects is still unclear. On mature endothelial cells, the cell surface F_1-ATP synthase has been previously characterized as a high affinity receptor of apoA-I, whereas the scavenger receptor SR-BI mainly binds with fully lipidated HDL and displays a poor affinity for lipid-free apoA-I. Furthermore, it was shown that apoA-I binding to surface ATP synthase on mature endothelial cells promotes cell proliferation, whereas inhibits apoptosis. In this work, we aimed to determine the effect of apoA-I in the proliferation and the angiogenic capacity of early hEPC, and the contribution of the cell surface ATP synthase in these events.

Results: We first evidenced that early hEPC express the ATP synthase at the surface of nonpermeabilized cells, where it is not colocalized with MitoTracker, a mitochondria marker. ApoA-I (50 μg/mL) increases hEPC proliferation ($+14.5\%$, $p < 0.001$) and potentiates the effect of hEPC on a cellular model of angiogenesis, with an increase of $+31\%$ ($p < 0.01$) in branch point counting and in tubule length. These effects of apoA-I were totally reversed in the presence of ATP synthase inhibitors, such as IF_1 or oligomycin, whereas the inhibition of the HDL receptor, SR-BI, partially inhibits these events.

Conclusions: These results provide the first evidence that surface ATP synthase is expressed on early hEPC, where it mediates apoA-I effects in hEPC proliferation and in angiogenesis. This knowledge could be helpful for future investigations focused on the regulation of the number and functionality of these cells and in the development of new therapies for the treatment of diseases, such as cardiovascular disease.

Introduction

Human endothelial progenitor cells (hEPC) are a subpopulation of stem cells present in peripheral blood, which can differentiate into endothelial lineage under a favorable medium (Asahara et al., 1997). In response to angiogenic stimuli, some cytokines, such as vascular endothelial growth factor (VEGF) and stromal-derived factor 1 (SDF-1), are secreted into the blood. These molecules can reach the bone marrow, where they induce the release of hEPC precursors (Heissig et al., 2002). These cells enter into the bloodstream, and then they are recruited at the sites of damaged endothelium, where they differentiate into mature endothelial cells and participate in reendothelialization and neovascularization processes (Cheng et al., 2013; Cui et al., 2011). For this reason, the number and functionality of hEPC, i.e., capacity to form endothelial colony units, neovascularization in response to ischemia and the repair of injured or damaged endothelium, are considered as protective factors against cardiovascular disease development (Capobianco et al., 2010; Hill et al., 2003; Werner et al., 2007).

There are early and late stages of hEPC differentiation towards an endothelial phenotype. Early hEPC express immaturity markers, such as CD34 and CD133, whereas markers of mature endothelial cells, such as vascular endothelium growth factor receptor 2 (VEGFR-2 or KDR), are expressed in a lesser extent. In addition, early hEPC display a round morphology, a low proliferative capacity and a high angiogenic potential (Hristov et al., 2003; Mukai et al., 2008). Conversely, late hEPC express endothelial cell markers, such as von Willebrand factor, E-selectin, VE-cadherin, VEGFR-2, and CD31, as they show low expression

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of immaturity markers. Furthermore, they have spindle morphology, high mitotic rate, and display the ability to incorporate into endothelial cell monolayers and form capillary-like structures (Hristov et al., 2003; Mukai et al., 2008). Thus, depending on the differentiation stage, hEPC exert different functions in vascular repairing and vasculogenesis.

High-density lipoproteins (HDL) are known because of their atheroprotective functions, which have been attributed to their central role in reverse cholesterol transport. However, HDL generate other protective effects associated to their direct interactions on the vascular wall, displaying antioxidant, anti-inflammatory, antithrombotic and cytoprotective properties (Mineo et al., 2006). In addition, it has been demonstrated that the concentration of HDL in the plasma is one of the most important factors determining hEPC levels in the bloodstream (Rossi et al., 2010) as well as hEPC functionality (proliferation, migration differentiation, angiogenesis) (Sumi et al., 2007; Zhang et al., 2010). On a mice model of allograft vasculopathy with elevated levels of HDL-cholesterol, these beneficial effects of HDL on EPC biology have been attributed to the scavenger receptor SR-BI, which is an HDL receptor expressed on EPC (Feng et al., 2009). However, other receptors might be involved and particularly those that would also bind the major HDL protein, apolipoprotein A-I (apoA-I) in its lipid-poor form, which represents about 5% of total plasma apoA-I concentration (i.e., 50–100 μg/mL). Accordingly, it has been reported that apoA-I mimetic peptides increase the number and functionality (proliferation, migration, and tube formation) of mouse (Yang et al., 2013) and human EPC (Z. Zhang et al., 2012).

A high affinity receptor for apoA-I, either as lipid-free or associated to HDL, called surface ATP synthase (SATP) or ecto-F_1-ATPase, has been described at the cell surface of mature endothelial cells (Radojkovic et al., 2009). This enzyme, responsible of 70% of apoA-I binding to the surface of mature endothelial cells (Radojkovic et al., 2009), is an entity similar to mitochondrial F_1F_O-ATP synthase but expressed ectopically at the surface of endothelial cells. ApoA-I binding stimulates the hydrolyze activity of ecto-F_1-ATPase (ATP conversion into ADP and inorganic phosphate), generating extracellular ADP (Cavelier et al., 2012; Radojkovic et al., 2009). This favors proliferation and reduces endothelial cell apoptosis through the activation of ADP-sensitive receptor(s) whose identity remains unknown (Radojkovic et al., 2009) and promotes HDL transcytosis through the activation of $P2Y_{12}$ receptor (Cavelier et al., 2012). Conversely, the IF_1 protein, a natural inhibitor of F_1-ATPase activity, reverses the protective effects of apoA-I on endothelial cells (Radojkovic et al., 2009).

The aim of this study was to determine the expression of SATP on early hEPC and its involvement in the effects of apoA-I on the proliferation and the angiogenic capacity of early hEPC.

Materials and methods

Materials

Ficoll-Histopaque reagent, oligomycin, and VEGF were provided by Sigma-Aldrich. EPC medium, M-199 medium, the antibody against the β chain of ATP synthase (A21351) and alamarBlue reagent were supplied by Invitrogen. Anti-CD34-PE antibody (550751), fibronectin, and Matrigel were from BD Biosciences, and anti-KDR-FITC antibody (FAB357F) was from R&D Systems. MitoTracker Red was from Molecular Probes. ECV-304 cells were provided by ATCC. The minimal inhibitory sequence of human IF_1 was generated as described (van Raaij et al., 1996).

Isolation and culture of early hEPC

hEPC were obtained as previously described (Asahara et al., 1997; Guzmán-Gutiérrez et al., 2010) from healthy volunteers (3 men and 3 women, 20–25 years old), who signed an informed consent. This protocol was carried out in accordance to the Declaration of Helsinki. Peripheral blood mononuclear cells (PBMC) were isolated from each donor by gradient centrifugation using Ficoll-Histopaque, and then they were separately cultured on fibronectin-coated dishes, using EPC medium supplemented with 30% FBS, VEGF 20 ng/mL, and penicillin 100 U/mL and streptomycin 100 μg/mL, at 37 °C in 5% CO_2. After 3 days, adherent cells, which correspond to early hEPC (Asahara et al., 1997; Guzmán-Gutiérrez et al., 2010), were recovered with PBS buffer/EDTA 1 mM. hEPC from different donors were plated separately for further experiments.

Culture of ECV-304

ECV-304 endothelial cells were grown as described (Q.H. Zhang et al., 2012) in M-199 medium supplemented with 10% new born calf serum, penicillin 100 U/mL and streptomycin 100 μg/mL, at 37 °C in 5% CO_2. Cells were cultured to about 80% confluence, and then they were recovered with trypsin/EDTA solution and plated for experiments.

HDL and apoA-I isolation

Total plasma samples were obtained from healthy volunteers (20–25 years old) who signed an informed consent, in accordance to the Declaration of Helsinki. HDL were isolated by ultracentrifugation as described (Havel et al., 1955). HDL were delipidated and apoA-I was isolated by a Sephadex G-150 size exclusion chromatography, using Tris–HCl 10 mM pH 8.0, NaCl 154 mM, EDTA 2.7 mM, and urea 7 M as elution buffer.

Flow cytometry

Early hEPC were incubated with a blocking solution (PBS 1% BSA) and labeled with anti-CD34-PE and anti-KDR-FITC antibodies. In another set of experiments, cells were incubated with a monoclonal antibody against the β chain of F_1-ATP synthase. After washes, a secondary anti-mouse IgG antibody conjugated to FITC was added for 1 h at 4 °C. Cells (1×10^5) were analyzed in a FACS Canto II (Becton-Dickenson, NJ), using the FACS Dive software.

Confocal microscopy

Nonpermeabilized hEPC were incubated with MitoTracker Red 10 mM for 30 min before fixation (4% PFA). Cells were saturated with PBS buffer/10% FBS and incubated for 1 h at 4 °C with a monoclonal antibody against the β chain of ATP synthase. A secondary anti-mouse IgG antibody conjugated to FITC was added for 1 h at 4 °C. When required, permeabilization was carried out by incubating cells with PBS 0.2% Triton X-100 solution for 45 min at room temperature, as described (Radojkovic et al., 2009). Pictures were captured using a Zeiss 710 LMS confocal microscope equipped with a 63× Plan-aprochromat objective.

Proliferation assays

Early hEPC were cultured in 96-well dishes in EPC medium supplemented with 30% FBS for 24 h and then with EPC medium 10% FBS for 4 h. ApoA-I or HDL were incubated for 48 h and cell number was assessed with the alamarBlue reagent, following the recommendations by the manufacturer. When required, inhibitors were added together with apoA-I.

In vitro angiogenesis

hEPC were added with ECV-304 cells (10,000:5,000) in 96-well dishes coated with Matrigel, in EPC medium supplemented with 10% FBS, which contained apoA-I or HDL. After 10 h, images were captured and the total area, the branch point number and the total length of capillary-like structures were quantified using ImageJ software, with the Angio J Matrigel Assay plugin, as described (Hofmann et al., 2012; Khoo et al., 2011). When required, inhibitors were added to the cultures together with apoA-I.