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Cardiac atrial appendage stem cells promote angiogenesis *in vitro* and *in vivo*

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ABSTRACT

Cardiac atrial appendage stem cells (CASCs) show extraordinary myocardial differentiation properties, making them ideal candidates for myocardial regeneration. However, since the myocardium is a highly vascularized tissue, revascularization of the ischemic infarct area is essential for functional repair. Therefore, this study assessed if CASCs contribute to cardiac angiogenesis *via* paracrine mechanisms.

First, it was demonstrated that CASCs produce and secrete high levels of numerous angiogenic growth factors, including vascular endothelial growth factor (VEGF), endothelin-1 (ET-1) and insulin-like growth factor binding protein 3 (IGFBP-3). Functional *in vitro* assays with a human microvascular endothelial cell line (HMEC-1) and CASC CM showed that CASCs promote endothelial cell proliferation, migration and tube formation, the most important steps of the angiogenesis process. Addition of inhibitory antibodies against identified growth factors could significantly reduce these effects, indicating their importance in CASC-induced neovascularization. The angiogenic potential of CASCs and CASC CM was also confirmed in a chorioallantoic membrane assay, demonstrating that CASCs promote blood vessel formation *in vivo*.

In conclusion, this study shows that CASCs not only induce myocardial repair by cardiomyogenic differentiation, but also stimulate blood vessel formation by paracrine mechanisms. The angiogenic properties of CASCs further strengthen their therapeutic potential and make them an optimal stem cell source for the treatment of ischemic heart disease.

1. Introduction

Ischemic heart disease is still one of the major causes of global morbidity and mortality since current therapies are not able to repair the damaged heart muscle [4]. Recent developments in stem cell biology and regenerative medicine show promise to replace the lost myocardium with functional healthy tissue [27]. Various stem cell types such as induced pluripotent stem cells [38], mesenchymal stem cells (MSCs) [18] and cardiac stem cells (CSCs) [2,40] have shown potential to improve heart function after myocardial infarction (MI). Most beneficial effects observed so far were mediated by paracrine actions, as stem cells secrete cytokines, growth factors and miRNAs that promote cardioprotection, angiogenesis and activate resident CSCs. However, only moderate therapeutic effects were observed in clinical trials. This can be explained by limited differentiation of these stem cells towards cardiomyocytes [15].

Abbreviations: ALDH, aldehyde dehydrogenase; ANG, angiogenin; Ang-1, angiopoietin-1; CAM, chorioallantoic membrane; CASC, cardiac atrial appendage stem cell; CM, conditioned medium; CSC, cardiac stem cell; DAPI, 4′,6-diamidino-2-phenylindole; DPPIV, dipeptidyl peptidase 4; DSC, dental stem cell; EC, endothelial cell; GM-CSF, granulocyte-monocyte colony stimulating factor; HMEC-1, human microvascular endothelial cell line 1; ELISA, enzyme-linked immunosorbent assay; EPC, endothelial progenitor cell; ET-1, endothelin 1; FBS, fetal bovine serum; IGFBP-1/2/3, insulin-like growth factor binding protein 1/2/3; IL-8, interleukin 8; MCP-1, monocyte chemotactic protein 1; MI, myocardial infarction; MSC, mesenchymal stem cell; MTT, 3-(4, 5-dimethylthiazolyl-2)-2.5-diphenyltetrazolium bromide; Neg Contr, negative control; Pos Contr, positive control; P/S, penicillin/streptomycin; PTX-3, pentraxin 3; TIMP-1, tissue inhibitor of metalloproteinase 1; uPA, urokinase plasminogen activator; VEGF, vascular endothelial growth factor.

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Recently, our research group identified a new CSC type in the adult human heart based on high aldehyde dehydrogenase (ALDH) enzyme activity, known as the cardiac atrial appendage stem cell (CASC) [22]. These CASCs are able to preserve left ventricular function in a Göttingen minipig infarction model based on extensive cardiomyogenic differentiation and functional integration [12,22]. Moreover, CASCs can be expanded to clinically relevant cell numbers [42], making them a perfect candidate for myocardial regeneration. However, to fully restore cardiac function, revascularization of the infarcted tissue is essential. Current catheter-based interventions and surgical bypass procedures are often not successful in reestablishing myocardial blood flow in MI patients, leading to an increased mortality and a poor clinical outcome [3]. In addition, cells transplanted in the infarct area end up in an ischemic environment, implicating that new blood vessel formation is necessary for their successful survival, engraftment and differentiation [36]. Ideally, cell-based therapies should therefore focus on both cardiomyogenesis and angiogenesis to induce optimal repair of the heart muscle. This study explores a possible contribution of CASCs in myocardial angiogenesis by investigating expression of pro- and *anti*-angiogenic factors and their role in three consecutive steps of the angiogenesis process (endothelial proliferation, migration, tube formation). Stimulation of angiogenesis would further strengthen the therapeutic potential of the CASCs in ischemic heart conditions.

2. Materials and methods

All procedures were carried out in accordance with the principles set forth in the Helsinki Declaration. Approval by the institutional review board and informed consent from each patient were obtained.

2.1. Cell culture

Atrial appendages were obtained from ischemic heart disease patients undergoing routine cardiac surgery and CASCs were isolated from atrial appendages based on a high ALDH enzyme activity as previously described [22]. Heart tissue was dissociated by collagenase type II treatment (600 U/ml; Invitrogen) and the obtained single cell suspension was stained with Aldefluor® (STEMCELL Technologies Inc.) according to the manufacturers' instructions. ALDH + cells were directly flow-sorted in X-vivo 15 supplemented with 10% fetal bovine serum (FBS) and 2% penicillin-streptomycin (P/S). The isolated CASCs were seeded in fibronectin-coated culture plates and expanded in X-vivo 15 medium supplemented with 20% FBS and 2% P/S. The medium was changed twice weekly and cells were re-plated at a density of 5×10^3 cells/cm^2 when reaching 80–85% confluence. After the first passage, serum levels were reduced to 10% and the cells were expanded. A human microvascular endothelial cell line (HMEC-1) was used to study angiogenesis *in vitro* [19] and was obtained from the Centre for Disease Control and Prevention (Atlanta, GA). HMEC-1 were cultured in MCDB 131 medium (Invitrogen, Carlsbad, *CA*) supplemented with 2% P/S, 10 mM L-glutamine (L-glut), 10% FBS, 10 ng/ml human epidermal growth factor (hEGF, Gibco, Paisley, UK) and 1 μg/ml hydrocortisone (HC, Sigma-Aldrich, Diegem, Belgium).

2.2. Preparation of conditioned medium and cell lysates

CASC conditioned medium (CM) and lysates were prepared from CASC cultures of passage 3 to 7, when reaching 85–90% confluence. After washing with phosphate buffered saline (PBS), the cells were cultured for 48 h in serum-free low glucose Dulbecco's modified eagle medium (LG-DMEM) 2% P/S 0% FBS. CM was harvested and passed through a 0.22 μm filter to remove cell debris. Afterwards the medium was concentrated in 3 kDa Amicon Ultra-15 centriprep tubes YM-10 (Amicon, Millipore Corp.) at 3600 g and sterile filtered to obtain 10× concentrated CM. Batches of 20× concentrated CM were also prepared for the *in vivo* chorioallantoic membrane (CAM) assay. In this way, a final concentration of 10× CM after 1:1 dilution in Matrigel was obtained. CASC viability was assessed after preparation of CM with an annexin V Kit to guarantee the quality of the CM (Number of viable CASCs >80%; Supplemental material). After CM collection, cell lysates were prepared by homogenization of CASCs in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1 mM sodium orthovanadate and protease inhibitor cocktail (1:100; Thermo Fischer Scientific, Erembodegem, Belgium) and stored at −80 °C until further analysis.

10× or 20× concentrated LG-DMEM 2% P/S 0% FBS served as negative control medium (Neg Contr) and LG-DMEM 2% P/S 10% FBS as positive control medium (Pos Contr), unless stated otherwise. For the functional HMEC-1 assays, CASC CM was pre-incubated with 500 ng/ml VEGF (R&D systems), 1 μg/ml IGFBP3 (R&D systems), 100 ng/ml ET-1 (Abcam) neutralizing antibody or isotype controls (ISO, R&D systems).

2.3. Human angiogenesis Array

A Proteome Profiler™ Human Angiogenesis Antibody Array (R&D Systems, Minneapolis, MN) was performed on CASC CM of 3 different patients and on negative and positive control media to identify angiogenic factors according to the manufacturer's instructions. Signal detection was performed using the LI-COR Odyssey Infrared Imaging System in combination with IRDye® 800CW streptavidin (Li-Cor, Westburg, The Netherlands). Quantification was performed with LI-COR Odyssey Image Studio analyzer software. Data were normalized against the positive control spots (Positive control was set to 100%, Blue demarcations in Fig. 1a).

2.4. Immunofluorescence for angiogenic growth factors

CASCs were cultured on glass coverslips and fixed in 4% paraformaldehyde (PFA) at 80% confluence. The cells were incubated overnight with a rabbit polyclonal *anti*-VEGF (1:100; R&D systems), a rabbit *anti*-IGFBP-3 (1:100; R&D systems) or a mouse *anti*-ET1 (1:100; Abcam) antibody at 4 °C in PBS 0.3% Triton. An Alexa Fluor 594 goat *anti*-rabbit antibody or an Alexa Fluor 555 goat anti-mouse antibody (1:500; life technologies) was used as secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Pictures were taken with a Nikon Eclipse 80i fluorescent microscope and a Nikon DS-2MBWc digital camera.

2.5. Western blot

After heat denaturation, protein lysates were separated on 4–15% Protean® TGX™ Gels and transferred to 0.2 μm Transblot® Turbo™ nitrocellulose membranes with the Transblot® Turbo™ Transfer System (all from Bio-rad, Temse, Belgium). Blots were blocked in Odyssey blocking buffer (Li-Cor) and primary antibodies used for western blot analysis included a rabbit *anti*-ET1 antibody (1:500; Abcam), a rabbit *anti*-IGFBP-3 antibody (1:300; Santa Cruz Biotechnology, Heidelberg, Germany) and a rabbit anti-VEGF antibody (1:300; Santa Cruz Biotechnology). A mouse *anti*-alpha-tubulin antibody (1:5000, Abcam) was used for the loading control. Secondary antibodies were a goat polyclonal *anti*-rabbit IR-Dye 800CW and a goat polyclonal anti-mouse IRDye 680CW antibody (1:15,000; Li-Cor). Detection was performed using an Odyssey Infrared Imaging System (Licor).

2.6. Enzyme-linked immunosorbent assay

The concentration of identified angiogenic factors was determined by ELISA for the negative and positive control, CASC CM and CASC lysates. Quantikine ELISA kits against ET-1, IGFBP-3 and VEGF (R&D Systems) were used according to the manufacturer's instructions.