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Pathophysiological aldosterone levels modify the secretory activity of cardiac progenitor cells

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ABSTRACT

Cardiac progenitor cells (CPCs) trigger regenerative processes via paracrine mechanisms in response to changes in their environment. In the present study we explored alterations in the secretory activity of CPCs induced by raised aldosterone levels symptomatic for heart failure. The cytokine profile of the supernatant of CPCs that were treated with the mineralocorticoid showed an induction of interleukin-6 secretion. Mass spectrometric analyses revealed an increase in the abundance of secreted proteins associated with regeneration and cell migration like gelsolin and galectin-1. Differential regulation of proteins associated with the extracellular matrix further points to an activation of cell migration. In response to supernatant, migration and proliferation were induced in CPCs, indicating a potential role of paracrine factors in the activation of CPCs from other regions of the heart or extra-cardiac sources. Changes in the secretory activity of CPCs might aim to compensate for the detrimental actions of aldosterone in heart failure.

1. Introduction

While clinical studies indicate that cell-based therapies might be useful for heart failure therapy [Assmus et al., 2014, Cheng et al., 2013, Fisher et al., 2014, Hong and Bolli, 2014, Tian et al., 2014], the underlying mechanisms by which these cells convey their protective role in the diseased tissue remain unclear [Rokosh et al., 2013]. Initially, injected stem cells were thought to directly compensate for the lost cardiomyocytes via trans-differentiation but various studies showed that the number of newly formed cardiomyocytes under cell-based therapies is rather low [Zimmermann and Eschenhagen, 2005]. Currently, the secretion of factors that induce regenerative processes including the attraction of endogenous stem cells is believed to be one of the main beneficial mechanisms of stem cell therapy [Hong and Bolli, 2014].

It is known that cardiomyocytes and fibroblasts, as well as resident cardiac stem cells respond to changes in their local environment by secreting different proteins [Burchfield and Dimmeler, 2008, Gnecchi et al., 2008]. Identifying those factors within the injured milieu that regulate the protein secretion will be critical to optimize the regenerative potential of cardiac progenitor cells (CPCs) and to maximize the functional benefits from cell transplantation [Ly et al., 2009]. The whole spectrum of proteins that are being released into the extracellular space *in vivo* or into conditioned media *in vitro* by a specific cell type is known as the secretome [Tjalsma et al., 2000]. Analysing the secretome of a specific cell type under defined conditions may help to elucidate the influence of pathophysiological stimuli on the function and regenerative capacity of the investigated cells, e.g. the role of the cell type in the improvement of wound healing [Chen et al., 2008, Walter et al., 2010], improvement of cardiac function or cardiac repair [Burchfield and Dimmeler, 2008] through paracrine and/or autocrine mechanisms. Previous cell therapy studies indicate a functional benefit in myocardial regeneration from proteins that were secreted by myocytes, fibroblasts or CPCs in response to injury [Burchfield and Dimmeler, 2008, Korf-Klingebiel et al., 2015,

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Nguyen et al., 2010]. Secreted proteins like extracellular matrix (ECM) proteins (e.g. collagens, laminins and proteoglycans) or low abundant but highly bioactive proteins like growth factors, cytokines and ECM-related proteases play a key role in the maintenance of cell integrity and stability, cell mobilization and cell differentiation [Chen et al., 2008, Gnecchi et al., 2008, Nguyen et al., 2010, Ohnishi et al., 2007, Uemura et al., 2006].

Heart failure is associated with increased levels of cytokines and neurohormones. The levels of the mineralocorticoid aldosterone were shown to be elevated after myocardial infarction and in chronic heart failure [Mizuno et al., 2001, Satoh et al., 2002, Silvestre et al., 1999], causing interstitial myocardial fibrosis that leads to ventricular stiffness which in turn contributes considerably to the progression of heart failure. These deleterious effects of aldosterone are mostly mediated through an intracellular mineralocorticoid receptor (MR). The presence of a cardiac MR was previously demonstrated, thus establishing the heart as a mineralocorticoid-responsive organ [Lombes et al., 1992, Weber et al., 1991]. MR antagonists, e.g. spironolactone or eplerenone, competitively bind to the MR and extenuate hypertrophy, fibrosis and diastolic dysfunction in human heart disease [Ohtani et al., 2007, Pitt et al., 2003].

While pathophysiological aldosterone levels are commonly associated with adverse effects on heart function including the formation of oxygen radicals and increased diastolic and systolic calcium levels, chronic exposure to aldosterone were also found to improve the contractile function of cardiomyocytes in a heart failure milieu [Wenzel et al., 2010]. Likewise, we were recently able to show that aldosterone, when used as an attractant, promotes the migration of resident CPCs, thereby possibly contributing to cardiac regeneration [Könemann et al., 2015]. We hypothesize that aldosterone does not only function as an attractant for progenitor cells but also influences the secretion of proteins that at least temporarily support the regeneration of injured tissue by contributing to the regenerative capacity of CPCs in an autocrine and paracrine fashion.

Therefore, we investigated the impact of pathophysiological aldosterone concentrations on the secretory activity of resident CPCs in order to detect alterations in paracrine factors and behaviour of these cells in response to diseased conditions.

2. Materials & methods

2.1. Mice

Wild type Friend leukemia virus strain B (FVB) mice of either sex at the age of six to eight weeks were used for the experiments. All animal tests were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the U.S. NIH (NIH Publication no. 85-23, revised 1985).

2.2. Isolation and culture of Sca-1 positive progenitor cells from adult murine hearts

Sca-1+ cells were isolated from healthy mouse hearts using magnetic cell sorting according to an adapted version of previously published protocols [Oh et al., 2003, Samal et al., 2012]. For detailed information please refer to Supplementary Text S1. Sca-1+ cells were seeded at a cell density of 32.000 cells per cm^2 on 25 cm^2 culture flask and cultivated using Dulbecco's Modified Medium and Nutrient Mixture F-12 (DMEM/F12 3:1, Invitrogen, Darmstadt, Germany) supplemented with 20% FBS (*fetal bovine serum*, Invitrogen, Darmstadt, Germany) at 37 °C in humid air with 5% CO_2 until they reached 70% confluency (4 days). Medium (cytokine assay) or serum-free medium (0% FBS; secretome analysis) supplemented with aldosterone (100 nmol/l) or aldosterone and eplerenone (2.5 μmol/l) was added for the appropriate time points. Untreated cells served as a control.

2.3. Cytokine multiplex assay

In order to quantify the levels of various cytokines in the supernatant of untreated and aldosterone (100 nmol/l) treated Sca-1+ cells, a mouse *FlowCytomix* 10plex assay kit (eBiosciences, Frankfurt a.M., Germany) was used. This fluorescent bead-based immunoassay allows the quantitative detection of cytokines in a concentration range of ~1–20.000 pg/ml. The factors analysed were: GM-CSF (*granulocyte macrophage colony-stimulating factor*), IFN-γ (*interferon* γ), interleukines IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-17 and TNF-α (*tumor necrosis factor*-α). The supernatant of either untreated or treated adherent Sca-1+ cells after 2 h, 24 h, 48 h and 72 h incubation time was subjected to centrifugation at 800 rpm for 10 min followed by filtration through a 0.22 μm filter to remove cell debris. Preparation of the samples and the standard curves were done according to the manufacturer's instructions. All samples were tested in duplicates and analysed using a fluorescence plate reader (PE (phycoerythrin) emission at 575 nm and far red emission at 700 nm). The calculation of the cytokine concentrations was carried out using the *FlowCytomix* Pro Software (eBiosciences).

2.4. Sample preparation for secretome analysis

After Sca-1+ cells reached 70% confluency they were washed twice with 1x PBS and once with serum-free medium, before fresh serum-free medium supplemented with aldosterone (100 nmol/l), eplerenone (2.5 μmol) or aldosterone (100 nmol/l) and eplerenone (2.5 μmol/l) was added. Controls were cultured in serum-free media only. Cells were incubated for another 2 h, 24 h or 48 h respectively, before the supernatant (5 ml) was collected and subjected to centrifugation at 800 rpm for 10 min followed by filtration using a 0.22 μm filter to remove cell debris. The proteins secreted were precipitated in the presence of 15% TCA (*trichloroacetic acid*) on ice for 1 h. Precipitates were harvested by centrifugation at 13,000 rpm for 15 min at 4 °C and then washed twice with ice-cold acetone. The protein pellet was air dried and subsequently resuspended in 2 mol/l urea - 0.5 mol/l thiourea (Sigma-Aldrich, Saint Louis, Missouri, U.S.A.). Protein content was determined using a Bradford assay kit (Pierce, Thermo Scientific, Bonn, Germany). For details of LC-ESI-MS/MS analysis, protein identification, quantification and functional classification please refer to Supplementary Text S2.

2.5. ELISA

Sca-1+ cells (n = 3) were transferred to a 12-well plate at a concentration of 1×10^4 cells per well. After Sca-1+ cells reached 70% confluency they were washed twice with 1x PBS and once with serum-free medium, before fresh serum-free medium alone or supplemented with aldosterone (100 nmol/l) was added. Cells were incubated for another 2 h, 24 h or 48 h respectively. Afterwards, the supernatant (800 μl) was collected, centrifuged at 800 rpm for 10 min and passed through a 0.22 μm filter in order to remove cell debris. Galectin-1 and gelsolin were measured in the supernatant using a Galectin-1 mouse ELISA Kit (Abcam, Cambridge, UK) and a mouse Gelsolin ELISA Kit (Cusabio, Biozol Diagnostica, Eching, Germany) following the manufacture's recommendations.