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Original article

Mesenchymal stem cell secreted platelet derived growth factor exerts a pro-migratory effect on resident Cardiac Atrial appendage Stem Cells

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ABSTRACT

Mesenchymal stem cells (MSCs) modulate cardiac healing after myocardial injury through the release of paracrine factors, but the exact mechanisms are still unknown. One possible mechanism is through mobilization of endogenous cardiac stem cells (CSCs). This study aimed to test the pro-migratory effect of MSC conditioned medium (MSC-CM) on endogenous CSCs from human cardiac tissue. By using a three-dimensional collagen assay, we found that MSC-CM improved migration of cells from human cardiac tissue. Cell counts, perimeter and area measurements were utilized to quantify migration effects. To examine whether resident stem cells were among the migrating cells, specific stem cell properties were investigated. The migrating cells displayed strong similarities with resident Cardiac Atrial appendage Stem Cells (CASCs), including a clonogenic potential of ~21.5% and expression of pluripotency associated genes like *Oct-4*, *Nanog*, *c-Myc* and *Klf-4*. Similar to CASCs, migrating cells demonstrated high aldehyde dehydrogenase activity and were able to differentiate towards cardiomyocytes. Receptor tyrosine kinase analysis and collagen assays performed with recombinant platelet derived growth factor (PDGF)-AA and Imatinib Mesylate, a PDGF receptor inhibitor, suggested a role for the PDGF-AA/PDGF receptor α axis in enhancing the migration process of CASCs. In conclusion, our findings demonstrate that factors present in MSC-CM improve migration of resident stem cells from human cardiac tissue. These data open doors towards future therapies in which MSC secreted factors, like PDGF-AA, can be utilized to enhance the recruitment of CASCs towards the site of myocardial injury.

1. Introduction

In the last decade, stem cell therapy has emerged as an innovative approach to restore cardiac function after myocardial infarction (MI) either directly by regeneration of functional myocardium [1] or indirectly by paracrine actions stimulating cardiac tissue healing [2]. Previously, researchers reported the existence of cardiac stem cells (CSCs) residing in the adult mammalian heart [3,4]. While phase 1 clinical studies [5,6] are completed only recently, CSC transplantations performed in the past already showed an improved cardiac function in animal models through regeneration of the damaged myocardium [7].

In the past, most experimental and clinical studies concerning ischemic heart disease (IHD) were performed with bone marrow stem cells (BM-SCs) [8,9]. Although it was demonstrated that BM-SC implantation can reduce ventricular remodeling and improve left ventricular function after MI, the underlying mechanism is still under debate [10]. Recent data propose that among BM-SCs, mesenchymal stem cells (MSCs) are especially capable of mediating cardiac repair through the release of a broad spectrum of cytokines, growth factors and chemokines into the damaged tissue area [11]. Strong evidence comes from studies that have utilized the conditioned medium derived from MSCs (MSC-CM).

Abbreviations: 3D, Three-dimensional; ALDH, Aldehyde dehydrogenase; AT, Annealing temperature; AxlR, Axl receptor; BM-SCs, Bone marrow stem cells; CASCs, Cardiac Atrial appendage Stem Cells; CFU-Fs, Colony-forming-unit fibroblasts; CSCs, Cardiac stem cells; cTn, Cardiac troponin; DEAB, Diethylamino-benzaldehyde; EGFR, Epidermal growth factor receptor; ELISA, Enzyme-linked immunosorbent assay; FAK, Focal adhesion kinase; FCS, Fetal calf serum; FGFR, Fibroblast growth factor receptor; FMO, Fluorescence minus one; GFP, Green fluorescent protein; IHD, Ischemic heart disease; InsR, Insulin receptor; LG-DMEM, Low-glucose Dulbecco's Modified Eagle Medium; MI, Myocardial infarction; MMPs, Matrix metalloproteinases; MSCs, Mesenchymal stem cells; MSC-CM, Mesenchymal stem cell conditioned medium; NRCMs, Neonatal rat cardiomyocytes; PDGF, Platelet derived growth factor; PDGFR, Platelet derived growth factor receptor; PLC-γ, Phospholipase C-γ; PI3K, Phospho-inositide-3 kinase; P/S, Penicillin/streptomycin; RALDH2, Retinaldehyde dehydrogenase 2; RTK, Receptor tyrosine kinase; RT-PCR, Reverse transcriptase PCR; Tβ4, Thymosin β4; Tbx18, T-Box 18; TGF-β, Transforming growth factor-β; VEGF, Vascular endothelial growth factor; VEGFR, Vascular endothelial growth factor receptor; Wt1, Wilm's tumor 1.

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Indeed, using in vitro assays and small animal models of MI, researchers have found that the administration of concentrated MSC-CM significantly improves myocardial regeneration and ventricular function [12]. Proposed mechanisms of action include the enhancement/modulation of cytoprotection [13], neovascularization [14], contractility [15], fibrotic remodeling [16] and inflammatory processes [17]. One proposed mechanism of paracrine influence, which has to date received little attention, is the mobilization of CSCs towards the injured site. To this end, the most important therapeutic goal is to stimulate CSCs to form cardiomyocytes and vascular cells to repopulate and regenerate the injured tissue.

In this study, we postulate that migration of endogenous stem cells from cardiac tissue can be enhanced by specific factors secreted by MSCs. To address our hypothesis, we directly assessed the effect of MSC-CM on human cardiac tissue fragments cultured inside a three-dimensional (3D) collagen matrix. We showed that MSCs secrete factors that improve migration of resident Cardiac Atrial appendage Stem Cells (CASCs), that are characterized by high levels of aldehyde dehydrogenase (ALDH) expression [4]. Further results indicated that the platelet derived growth factor receptor α (PDGFRα) plays an important role in the migration process. These findings open new perspectives to stimulate cardiac tissue healing via activation of endogenous repair mechanisms.

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. All animal studies were approved by the Hasselt University Institutional Animal Care and Use Committee.

A detailed version of Materials and methods is available in the Suppl. Material.

2.1. Preparation of MSC-CM

Human bone marrow MSCs were obtained as previously described [18]. Once MSCs of at least passage 4 (P4) reached 70–75% confluence, medium was replaced with low-glucose Dulbecco's Modified Eagle Medium (LG-DMEM; Invitrogen) containing 10% fetal calf serum (FCS: Hyclone) and 2% penicillin/streptomycin (P/S; Lonza) until MSC-CM was prepared. MSC-CM was prepared from 85 to 90% confluent T175-cm^2 flasks containing 1.5×10^6 MSCs as described [19]. MSC-CM at 10× concentration was utilized, unless stated otherwise.

2.2. Collagen migration assay

Migration assays were adapted from experiments performed by De Wever et al. with minor modifications [20]. Under a macroscope with a calibrated ocular grid (Wild M5, Wild Heerbrugg), atrial appendages were cut into ±400 μm^3 fragments and suspended in collagen type I solution (1 mg/ml; Becton&Dickinson). Depending on the type of experiment, either 5×10^5 MSCs were plated on the bottom of the well or 1 ml of the appropriate medium was administered on top of the matrix. Where only medium was administered, 1 ml of serum-free LG-DMEM served as negative control medium. Tested media consisted of 1 ml of prepared MSC-CM or 1 ml of recombinant platelet derived growth factor (PDGF)-AA (R&D Systems) dissolved in serum-free LG-DMEM at a concentration of 0.1 ng/ml, 1 ng/ml or 100 ng/ml. Additionally, inhibition experiments were performed in which 1 μM of the multi-target inhibitor Imatinib Mesylate (Selleckchem) was added to control, MSC-CM or PDGF-AA conditions, as indicated. Alternatively, to block receptor binding by PDGF-AA, a neutralizing PDGFRα antibody (R&D systems) was added to control and MSC-CM at a final concentration of 20 ng/ml. Cellular migration was regularly scored during a total period of at least 10 days. Per test condition, minimal 20 randomly selected tissue fragments were analyzed. The number of migrating cells was counted using an Axiovert 200 M microscope (Zeiss). Perimeter and area measurements were performed on the evasion zone from the tissue fragments with Axiovision 4.8 software (Zeiss) and utilized as measures of migration. Tissue viability was assessed at the end of the follow-up using an Annexin V staining kit (Becton&Dickinson) according to the manufacturer's instructions.

2.3. Isolation and expansion of migrating cells

To isolate migrating cells, cardiac tissue pieces were first removed from the collagen matrix. The remaining collagen was dissolved by collagenase type II treatment (600 U/ml; Invitrogen) at room temperature for 10 min. Cells were seeded in a 96-well plate and expanded in X-Vivo 15 medium containing 20% FCS and 2% P/S. After P1, serum level was reduced to 10%.

2.4. Cell cycle analysis of migrating cells

For analysis of cell cycle distribution, the BD Cycletest™ Plus DNA Reagent Kit (Becton& Dickinson) was utilized, in accordance with the manufacturer's instructions. To obtain a mitotic arrest at the metaphase, 10 ng/ml KaryoMAX® Colcemid™ Solution (Invitrogen) was added for 48 h. Cellular DNA content was monitored on a FACSCanto® (Becton&Dickinson). Cells in S + G_2M phase were judged to be actively proliferating cells. Mitotic indices were calculated with ModFit LT software 3.0 (Verity Software House).

2.5. Clonogenic assay

Prior to assess the clonogenic character, migrating cells were labeled with green fluorescent protein (GFP). Subsequently, a total of 864 GFP$^+$ cells (P5-P7) were flow sorted with a FACSAria® at a density of 1 cell/well in X-Vivo 15 medium containing 20% FCS. Single-cell deposition was confirmed by fluorescence microscopy and wells containing more than one cell were excluded. Colonies were scored after 10 days.

2.6. Expression of pluripotency associated genes

Total RNA was isolated from expanded migrating cells (P3–P5) using the RNeasy Mini kit (Qiagen). cDNA was synthesized using Superscript III and random hexamers (Invitrogen). Reverse transcriptase PCR (RT-PCR) using Taq polymerase (Roche) was performed for 35 cycles consisting of 40 s at 95 °C, 50 s at annealing temperature (AT) and 1 min at 72 °C with a final extension step of 10 min at 72 °C. β-Actin was used as control. Primer sequences with corresponding AT and expected fragment size are listed in Suppl. Table 1.

2.7. Analysis of ALDH activity

ALDH expression of migrating cells was analyzed with the Aldefluor™ kit (Aldagen, Inc). Cells were seeded in 24-well plates at 1×10^4 cells/well and incubated in 500 μl Aldefluor assay buffer containing 2.5 μl of activated Aldefluor® (Aldagen, Inc). To confirm specificity for ALDH, 20 μl of diethylamino-benzaldehyde (DEAB) was administered to block ALDH activity. After incubation at 37 °C for 30 min, cells were washed and kept in Aldefluor assay buffer for microscopical visualization for the green fluorescent reaction product. Exposure times were kept constant during each acquisition.

2.8. Flow cytometrical analysis

The antigen expression profile of migrating cells and MSCs was determined by flow cytometry. 5×10^4 cells/tube were incubated for 20 min in the dark with human monoclonal antibodies as recommended by the manufacturer.