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Original article

Donor cell-type specific paracrine effects of cell transplantation for post-infarction heart failure

Yasunori Shintani [a], Satsuki Fukushima [b], Anabel Varela-Carver [b], Joon Lee [b], Steven R. Coppen [a], Kunihiko Takahashi [a], Scott W. Brouilette [a], Kenta Yashiro [a], Cesare M.N. Terracciano [b], Magdi H. Yacoub [b], Ken Suzuki [a,b,*]

[a] Translational Cardiovascular Therapeutics, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London, UK
[b] Harefield Heart Science Centre, National Heart and Lung Institute, Faculty of Medicine, Imperial College London, UK

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ABSTRACT

Cell transplantation is an emerging therapy for treating post-infarction heart failure. Although the paracrine effect has been proposed to be an important mechanism for the therapeutic benefits, details remain largely unknown. This study compared various aspects of the paracrine effect after transplantation of either bone marrow mononuclear cells (BMC) or skeletal myoblasts (SMB) into the post-infarction chronically failing heart. Three weeks after left coronary artery ligation, adult rats received intramyocardial injection of either BMC, SMB or PBS only. Echocardiography demonstrated that injection of either cell type improved cardiac function compared to PBS injection. Interestingly, BMC injection markedly improved neovascularization in the border areas surrounding infarcts, while SMB injection decreased fibrosis in both the border and remote areas. Injection of either cell type similarly reduced hypertrophy of cardiomyocytes as assessed by cell-size planimetry using isolated cardiomyocytes. Quantitative RT-PCR revealed that, among 15 candidate mediators of paracrine effects studied, *Fgf2* and *Hgf* were upregulated only after BMC injection, while *Mmp2* and *Timp4* were modulated after SMB injection. Additional investigations of signalling pathways relevant to heart failure by western blotting showed that p38 and STAT3 were temporarily activated after BMC injection, in contrast, ERK1/2 and JNK were activated after SMB injection. There was no difference in activation of Akt, PKD or Smad3 among groups. These data suggest that paracrine effects observed after cell transplantation in post-infarction heart failure were noticeably different between cell types in terms of mediators, signal transductions and consequent effects.

1. Introduction

Recent progress in reperfusion therapy has dramatically decreased the early mortality of acute myocardial infarction (MI), while on the other hand the number of patients with post-MI chronic heart failure (HF) has been increasing considerably. Therefore, development of a new treatment for post-MI HF is a clinical priority and previous studies have shown that cell transplantation is a promising option [1]. This emerging therapy has, however, several fundamental issues, including uncertain mechanisms by which grafted donor cells improve performance of post-MI failing hearts. We and others have consistently described poor survival of injected cells in the heart as well as the extremely rare occurrence of cardiomyogenic differentiation of donor cells or fusion with host cardiomyocytes [1–3]. These data suggest that paracrine effects, rather than direct mechanical contribution, play a vital role in the cell therapy-induced benefits [4,5]. It has also been reported that cell transplantation attenuates post-MI adverse ventricular remodeling *via* paracrine effects by reducing cardiomyocyte apoptosis/hypertrophy, increasing neovascular formation and decreasing pathological accumulation of extracellular matrix [5–7].

Elucidation of the paracrine effects is therefore key to a comprehensive understanding and further refinement of cell transplantation for treating post-MI HF. A number of previous studies aimed to identify responsible paracrine molecules secreted from donor cells *in vitro* and *in vivo* [4,5,7,8] and have suggested that a variety of molecules including VEGF, FGF2, IL-10 and IGF-1 may contribute to cell therapy-induced paracrine effects. However, secretion by donor cells is likely to be different between *in vitro* and *in vivo* conditions. It should also be noted that there are many types of cells which will secrete paracrine molecules *in vivo*: not only the donor cells but also host cardiac cells and accumulated inflammatory cells. In addition, whereas most *in vivo* studies have used acute MI models, donor cells are likely to behave differently in chronically failing myocardium. Therefore, paracrine mechanisms of cell-transplantation-induced effects on post-MI HF

* Corresponding author. Translational Cardiovascular Therapeutics, William Harvey Research Institute, Barts and The London School of Medicine, Queen Mary University of London, Charterhouse Square, London EC1M 6BQ, UK. Tel.: +44 20 7882 8233; fax: +44 20 7882 8256.
E-mail address: ken.suzuki@qmul.ac.uk (K. Suzuki).

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remain largely unknown. Furthermore, although different types of donor cells are likely to generate distinct secretion profiles, there is no report that compares paracrine effects and underlying molecular mechanisms among different donor cells using *in vivo* models.

Upregulated paracrine mediators are presumed to affect cardiac cells by activating intracellular signalling pathways *via* relevant receptors, resulting in phenotypic changes. Previous studies have investigated molecules secreted from donor cells or upregulated within the myocardium, but none of these have assessed the signalling pathways activated after cell transplantation. It is important to be aware that upregulation of molecules detected by expression-based approaches may not be necessarily vital in the paracrine effect. If any inhibitory factors are upregulated at the same time, the signal of a molecule in the host tissues would be reduced or cancelled out. Conversely, accumulation of subtle changes in various interacting molecules, each of which is undetectable by current technologies, may activate important signalling pathways. Elucidation of the changes in downstream signalling molecules within the myocardium after cell transplantation will therefore be important in obtaining a comprehensive understanding of the paracrine effects.

In this study, we directly compared the paracrine effects after injection of bone marrow mononuclear cells (BMC) and skeletal myoblasts (SMB), using a rat post-MI chronic HF model, with particular focus on paracrine mediators, downstream signal transductions and consequent beneficial effects. Therapeutic effects of transplantation of BMC and SMB have been well described [1], whereas these cells have distinct cellular origin and properties. Therefore, comparison between these cell types will be useful to highlight the cell-type specific paracrine effects and mechanism.

2. Materials and methods

All studies were performed with approval of the institutional ethics committee and the UK Home Office. The investigation conforms to the Principles of Laboratory Animal Care (National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (National Institutes of Health). All procedures and evaluations, including assessment of cardiac parameters, were carried out in a blinded manner.

2.1. Generation of myocardial infarction and cell injection

Generation of post-MI HF by left coronary artery ligation and primary SMB and BMC isolation from male GFP transgenic Sprague–Dawley rats (200–250 g, Rat Research and Resource Centre) were carried out as previously described [2,9]. At 21 days after coronary artery ligation either SMB (5×10^6 cells suspended in 100 μl of PBS), BMC (10×10^6 cells suspended in 100 μl of PBS) or PBS (100 μl of PBS only) were injected directly into two sites of the infarct border zone [2,3]. The numbers of cells injected have been chosen based on the previous experimental and clinical reports, which showed positive therapeutic effects, from our and other laboratories [1]. As our previous studies have shown that survival of SMB is more than two-fold greater than that of BMC at 3 days following injection into the chronically failing hearts, we injected a larger number of cells for BMC. It has already been confirmed that this cell-transplantation model in Sprague–Dawley rats causes only negligible immune response [2,9].

2.2. Assessment of cardiac function

Echocardiography (Sequoia 512 and 15-MHz probe, Acuson) was serially performed as previously described ($n=10$ for each group) [2]. Left ventricular (LV) fractional area change (%FAC) was assessed as an indicator for LV systolic function with 2-D mode at the papillary muscle level. The rats with %FAC ≥ 30% at 1 day before cell injection were excluded.

2.3. Histological assessment

To evaluate capillary density in the border area, cryosections from day 28 samples were immunolabelled with anti-von Willebrand Factor antibody (1:250 dilution, DAKO) ($n=5$ in each group). The images were obtained by a confocal laser scanning microscopy (LSM510 system, Zeiss). The number of capillary vessels in the peri-infarct myocardium (16 individual fields per one heart) was counted under the high-power field (×400) and averaged to express a capillary density (per 1 mm^2).

To evaluate collagen deposition, cryosections from day 28 samples were stained with 0.1% picrosirius red ($n=5$ in each group). Collagen volume fraction in each section was assessed by analysing high-power field images in the border and remote areas using ImageJ software. Ten individual fields per heart were examined.

2.4. Cell isolation and cell area planimetry

Cardiomyocytes were isolated from the LV by using Langerdorff-perfused heart system [3,10,11]. Rats were sacrificed and the hearts were removed at day 28 after cell injection ($n=4$ in each group). After perfusion with enzyme solution, the infarct scar was discarded, and cardiomyocytes were isolated only from the remaining LV tissue. Overall cardiomyocyte viability was 70–80% and used within 6 h. A sufficient number of viable cardiomyocytes having a typical rod shape were randomly selected and examined using an Olympus IX-71 inverted epifluorescence microscope with a ×60 objective. Photographs were taken and the projected 2-dimensional area for each cell was measured using ImageJ software.

2.5. Quantitative Reverse-Transcriptase PCR

Total RNA was extracted from the whole LV using TRIzol (Invitrogen). After cleaning and DNase treatment using an RNeasy kit (Qiagen), cDNA was synthesised from 1 μg of the total RNA by using a reverse-transcriptase with random hexamer primers (Applied Biosystems). Quantitative Reverse-Transcriptase PCR was carried out using TaqMan technology using ABI Prism 7900 detection system (Applied Biosystems) according to the manufacturer's instruction. Each sample was analysed in triplicate for each genes studied ($n>4$ at each time point in each group). Data were normalised to the rodent glyceraldehyde phosphate dehydrogenase (*Gapdh*) expression level. For relative expression analysis we adopted the ddCT method, and referred to the PBS group.

2.6. Western blotting

Tissue homogenates from whole LV samples ($n=4$ to 6 each groups, at days 3 and 28) were prepared using lysis buffer (100 mM Tris pH 7.4, 20% SDS, 10 mM EDTA, 10 mM NaF, 2 mM sodium orthovanadate). Antibodies were obtained from Cell Signaling; anti-phosphorylated ERK1/2 (#4377), phosphorylated JNK (#9251), phosphorylated p38 (#9216), phosphorylated Akt (#4051), phosphorylated PKD (#2054), phosphorylated STAT3 (#9145), phosphorylated Smad3 (#9520). Labelled membrane was stripped, then re-probed with anit-ERK1/2 (#9102), JNK (#9252), p38 (#9212), Akt (#9272), PKD (#2052), STAT3 (#9132), Smad3 (#9523). Blots were scanned and quantitative analysis was performed using ImageJ software. The proportion of the phosphorylated protein to the total protein was normalised to that of the PBS group.

2.7. Statistical analysis

All values are expressed as means ± SEM. %FAC was assessed by two-way ANOVA repeated measure and all other statistics were assessed with one-way ANOVA using Bonferroni's post-hoc test. A value of $p<0.05$ was considered statistically significant.