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The use of cell-sheet technique eliminates arrhythmogenicity of skeletal myoblast-based therapy to the heart with enhanced therapeutic effects ☆

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ABSTRACT

Background: Clinical application of skeletal myoblast transplantation has been curtailed due to arrhythmogenicity and inconsistent therapeutic benefits observed in previous studies. However, these issues may be solved by the use of a new cell-delivery mode. It is now possible to generate "cell-sheets" using temperature-responsive dishes without artificial scaffolds. This study aimed to validate the safety and efficacy of epicardial placement of myoblast-sheets (myoblast-sheet therapy) in treating heart failure.

Methods and results: After coronary artery ligation in rats, the same numbers of syngeneic myoblasts were transplanted by intramyocardial injection or cell-sheet placement. Continuous radio-telemetry monitoring detected increased ventricular arrhythmias, including ventricular tachycardia, after intramyocardial injection compared to the sham-control, while these were abolished in myoblast-sheet therapy. This effect was conjunct with avoidance of islet-like cell-cluster formation that disrupts electrical conduction, and with prevention of increased arrhythmogenic substrates due to exaggerated inflammation. Persistent ectopic donor cells were found in the lung only after intramyocardial injection, strengthening the improved safety of myoblast-sheet therapy. In addition, myoblast-sheet therapy enhanced cardiac function, corresponding to a 9.2-fold increase in donor cell survival, compared to intramyocardial injection. Both methods achieved reduced infarct size, decreased fibrosis, attenuated cardiomyocyte hypertrophy, and increased neovascular formation, in association with myocardial upregulation of a group of relevant molecules. The pattern of these beneficial changes was similar between two methods, but the degree was more substantial after myoblast-sheet therapy.

Conclusion: The cell-sheet technique enhanced safety and therapeutic efficacy of myoblast-based therapy, compared to the current method, thereby paving the way for clinical application.

1. Introduction

Despite pre-clinical evidence showing that transplantation of skeletal myoblasts (SMBs) greatly improves the function of damaged hearts mainly via the paracrine effect [1], the use of this cell type in clinical cell therapy has been largely curtailed. This was mainly due to two adverse findings in previous clinical studies: occurrence of fatal ventricular arrhythmias and insufficient or inconsistent therapeutic effects [1,2]. We speculate that these issues were associated with the use of a suboptimal cell-delivery method, and that application of a more suitable method may solve both of these concerns.

The commonly used cell-delivery method in previous studies is direct intramyocardial injection of trypsin-treated SMB suspensions [1,2]. This method is, however, known to produce islet-like localized cell-clusters, which could cause disturbance of the electrical conduction, leading to re-entrance arrhythmias [3–5]. In addition, this method is associated with considerable donor cell loss by initial leakage and by cell death/damage due to injection-mediated mechanical injury and subsequent myocardial inflammation. Additional donor cell damage is caused by the enzymatic digestion (i.e. trypsinization) used for cell collection from culture dishes [5–7]. Trypsinization disrupts cell surface proteins and destroys cell–cell connections, thus deteriorating donor cell viability and functionalities. These adverse effects would collectively result in poor donor cell engraftment, which will consequently limit the benefit from this approach [1].

Recent development of the unique culture dish coated with a temperature-responsive polymer (poly-N-isopropylacrylamide) has enabled fabrication of "cell-sheets" simply by reduction of the temperature without any harmful chemical treatment and without using artificial

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scaffolds [7]. At 37 °C this polymer is hydrophobic, and cells can adhere to the dishes and grow. However, when the temperature is dropped to 25 °C or below, the polymer rapidly becomes hydrophilic, hydrated and swollen, losing its cell-adhesiveness. As a result, the cells detach from the dish as a free cell-sheet. In contrast to trypsinization, cell surface proteins, cell–cell junctions and underpinning extracellular matrix (ECM) are well preserved in this method. Following epicardial placement, cell-sheets are expected to quickly adhere to the heart due to the preserved ECM, minimizing donor cell leakage. Taken together, the epicardial placement of SMB-sheets (SMB-sheet therapy) is likely to achieve greater retention, survival, and engraftment of donor SMBs in the heart while maintaining important donor cell functionalities including the secretion of paracrine mediators, resulting in augmentation of therapeutic benefits, compared to intramyocardial injection. In addition, this innovative method will not produce intramyocardial tissue disruption that disturbs the electrical conductance, and therefore might prevent occurrence of ventricular arrhythmias. In fact, therapeutic effects of SMB-sheet therapy have been reported in various models [8–10]. However, more detailed pre-clinical investigations, particularly on arrhythmia occurrence and other factors concerning the safety and effects, are needed for this approach to be widely established in the clinical arena.

2. Materials and methods

All animal studies were performed with the approval of the institutional ethics committee and the Home Office, UK. The authors of this manuscript have certified that they comply with the Principles of Ethical Publishing in the International Journal of Cardiology. All procedures were carried out in a blind manner whenever possible.

2.1. Generation of SMB-suspensions and SMB-sheets

SMBs were collected from male Lewis rats (150–175 g, Charles River) by the single fiber method as previously described [5,11]. To generate an SMB-sheet, 4×10^6 SMBs (passage 4–5) were seeded on a 35-mm temperature-responsive culture dish (UpCell, CellSeed, Inc.). Following 12–15 h incubation at 37 °C, the temperature was lowered to 22 °C, enabling the SMB-sheet to detach from the dish [8]. For injection, 4×10^6 SMBs were collected using trypsinization and suspended in 200 μl PBS [5]. The size of generated SMB-sheets was approximately 15 mm in diameter. For graft tracking studies, SMBs were labeled with CM-DiI (Molecular Probes) according to the manufacturer's protocol.

2.2. Induction of myocardial infarction (MI) and SMB transplantation

Female Lewis rats (180–200 g, Charles River) underwent left coronary artery ligation as previously described [3,5]. The animals were randomly assigned to receive either SMB-sheet therapy (Sheet group), intramyocardial SMB injection (IM group), or sham-treatment (Cont group). For the Sheet group, an SMB-sheet was epicardially placed to cover the left ventricular (LV) free wall including both infarct and border areas. For the IM group, SMB suspension was injected into 2 sites (100 μl each) of the LV free wall, aiming to target a similar area to SMB-sheet therapy [3,5].

2.3. Measurement of arrhythmia occurrence

Incidence of spontaneous arrhythmias, including premature ventricular contraction (PVC), ventricular tachycardia (VT) and ventricular fibrillation (VF), was continuously monitored by a radio-telemetry system (Data Sciences International) as described previously [3,5]. For accurate evaluation of the arrhythmia severity, the modified Curtis and Walker's scoring system [12] was applied, where frequencies of PVC, VT and VF were systematically taken into account.

2.4. Histological analysis

At chosen time points, the hearts were excised, fixed with 4% paraformaldehyde, and frozen. Cryosections were cut and incubated with polyclonal anti-cardiac troponin-T antibody (1:200 dilution, HyTest), biotin conjugated *Griffonia simplicifolia* lectin I-isolectin B_4 (1:100, Vector), monoclonal anti-CD45 antibody (1:50, BD), monoclonal anti-CD11b antibody (1:50, Chemicon), monoclonal anti-granulocyte antigen (1:20, AbD Serotec), monoclonal OX62 (1:25, AbD Serotec), polyclonal CD3 (1:100, Abcam), or monoclonal connexin43 (Cx43; 1:250, Millipore), followed by visualization using appropriate fluorophore-conjugated secondary antibodies with or without nuclear counter-staining using 4′,6-diamidino-2-phenylindole (DAPI). Ten different fields from each of the border and remote areas per heart were randomly selected and assessed. Another set of sections were stained with 0.1% picrosirius red for assessing infarct size and for detecting collagen deposition [3,5]. To evaluate the cardiomyocyte size, the cross-sectional area of appropriately detected cardiomyocytes [13] was measured of 50 cardiomyocytes in each border and remote area per heart.

2.5. Evaluation of cardiac performance

Cardiac function and dimensions, and hemodynamic parameters were measured by using echocardiography (Vevo-770, VisualSonics) and cardiac catheterization (SPR-320 and PVAN3.2, Millar Instruments) by a blinded operator as previously described [3,5,13].

2.6. Analysis for donor cell survival in the heart and other organs

DNA was extracted from the heart, lung, liver, kidney, and spleen post treatment. The presence of male cells in each female organ was quantitatively assessed to define donor cell presence using real-time PCR (Prism 7900HT, Applied Biosystems) for the Y-chromosome-specific *sry* gene as previously described [3,5]. Non-heart organs that were positively detected for *sry* expression were defined to be ectopic donor cell survival.

2.7. ELISA for myocardial IL-1β levels

Proteins were extracted from the homogenates from frozen whole LV samples collected at day 3 post-treatment with lysis buffer (0.15 M NaCl, 1 mM EDTA, 20 mM Tris pH 7.4, 1 mM DTT and protease inhibitor cocktail (Sigma)). After measuring the protein concentration (BioRad DC protein assay), levels of IL-1β were measured using an ELISA kit (eBioscience) according to the company's instruction.

2.8. Analysis for myocardial gene expression

Total RNA was extracted from frozen whole LV samples and assessed for myocardial gene expression of genes presumably relevant to the SMB-mediated paracrine effect by quantitative RT-PCR (Prism 7900HT) as previously described [14]. TaqMan primers and probes were purchased from Applied Biosystems. Expression was normalized using *Ubiquitin C*.

2.9. Statistical analysis

All values are expressed as mean ± SEM. Statistical comparison of the data was performed using the Student's unpaired *t*-test for the donor cell survival in the heart and using χ^2-test for the ectopic donor cells. Other data were analyzed with one-way ANOVA followed by Fisher's post-hoc analysis to compare groups. A value of $p<0.05$ was considered statistically significant.

3. Results

3.1. Eliminated arrhythmia occurrence by SMB-sheet therapy

We established a rat model to investigate whether the use of the cell-sheet technique might reduce arrhythmogenicity associated with SMB transplantation. Continuous electrocardiogram monitoring using radio-telemetry revealed frequent episodes of premature ventricular contractions (PVCs) in the IM group at both day 1 and 28 (Fig. 1A, C) consistent with previous clinical and experimental reports [1,5], validating the suitability of this model. There was a smaller number of PVC occurrence observed in the Cont group, which is also commonly seen post-MI [5,11], and more importantly the frequency of PVCs in the Sheet groups was just comparable to this base-line data. Furthermore, the IM group showed more frequent ventricular tachycardia (VT) occurrence than other groups; more than one-third of the animals in the IM group developed VT (Fig. 1B, D). Furthermore, one animal of the IM group developed transient ventricular fibrillation (VF) at day 28. In contrast, there was no animal that developed VT or VF in the Sheet group throughout the period studied. Consequently, the IM group, but not Sheet group, showed a higher arrhythmia score than the Cont group (Fig. 1E). These data are the first quantitative evidence that SMB transplantation-induced arrhythmogenicity can be prevented by the use of the cell-sheet technique.

3.2. Donor cell behaviors after SMB-sheet therapy

To gain an insight of the mechanism by which SMB-sheet therapy attenuated arrhythmogenicity, we first assessed the quantitative donor cell survival (presence). As a result, the donor cell presence in the heart in the Sheet group was 3.5-fold and 9.2-fold higher than that in the IM