A Factor Underlying Late-Phase Arrhythmogenicity After Cell Therapy to the Heart

Global Downregulation of Connexin43 in the Host Myocardium After Skeletal Myoblast Transplantation

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Background—Arrhythmia occurrence is a variable but serious concern of cell therapy for treating heart failure. Using a rat postinfarction chronic heart failure model, we compared skeletal myoblast (SMB) with bone marrow cell (BMC) injection to highlight donor cell-specific, late-phase arrhythmogenesis and the underlying factors.

Methods and Results—SMBs or BMCs derived from male GFP-transgenic rats, or PBS were injected intramyocardially into female rat hearts 3 weeks after coronary artery occlusion. At 28 days after injection, echocardiography showed that the left ventricular ejection fraction was significantly improved in both the SMB and BMC groups, compared to PBS control despite poor graft survival as assessed by PCR for the male-specific gene. Radio-telemetry analysis revealed that the SMB group displayed a higher occurrence of ventricular premature contractions with an elongation of the QRS complex and the hearts were more susceptible to isoproterenol-induced ventricular tachycardia compared to the BMC and PBS groups. Western blot and immunoconfocal analysis showed that the gap junction protein, connexin43, was widely and persistently decreased in the SMB group compared to the other groups. IL-1β was shown to be upregulated in hearts after SMB injection, and in vitro experiments demonstrated that exposure to IL-1β caused a decrease in connexin43 and intercellular communication in cultured cardiomyocytes.

Conclusions—Although cell therapy was capable of improving function of the postinfarction chronically failing heart, there was late-phase arrhythmogenicity specific to donor cell type. Global downregulation of connexin43 in the host myocardium was indicated to be an important factor underlying late-phase arrhythmogenicity after SMB transplantation.

Key Words: cell therapy ▪ skeletal myoblast ▪ arrhythmia ▪ connexins

Cell therapy using skeletal myoblasts (SMBs) and bone marrow cells (BMCs) is an emerging strategy for treating heart failure.1-5 However, clinical studies using intramyocardial SMB transplantation have shown highly variable incidences of ventricular tachycardia (VT) in both early and late phases after treatment.2-3,5 In contrast, there have been no clinical reports showing significant arrhythmogenicity after BMC transplantation. But, the arrhythmogenicity in these clinical trials could be masked or affected by the use of antiarrhythmia drugs, and/or influenced by the heterogeneous nature of the patients studied, requiring further research using appropriate models/patients to determine the “true” arrhythmogenicity of cell therapy.

We have recently shown that, in a drug-free rat model of postinfarction chronic heart failure, direct intramyocardial, but not intracoronary, injection of BMCs caused frequent ventricular arrhythmias in the early phase after cell therapy with a peak at 7 days and largely disappearing by 28 days.6 This was presumably because of local isolated cell clusters produced by intramyocardial cell injection, which would induce electrophysiological heterogeneity. On the other hand, previous clinical studies using SMBs demonstrated that arrhythmogenicity occurred even in the long term (>1 month) after cell therapy.2,3,5 It is likely that this type of late arrhythmia is related to the grafted cells rather than to the injection procedure. However, the pattern, degree, and factors responsible for such late-phase arrhythmia occurrence remain uncertain.

In this study, we compared SMB with BMC injection to highlight donor cell-specific, late-phase arrhythmogenesis...
and the underlying factors after cell therapy using a rat postinfarction chronic heart failure model.

Methods
Studies were carried out with the approval of the UK Home Office and conform to the Principles of Laboratory Animal Care (National Society for Medical Research) and the Guide for the Care and Use of Laboratory Animals (NIH). All surgical procedures and evaluations were carried out in a blinded manner.

Collection of Donor Cells
SMBs were isolated from the extensor digitorum longus of male GFP-transgenic Sprague-Dawley (SD) rats (200 to 250g. Rat Research & Resource Center, Columbia, Mo, USA) by the single fiber method and cultured as described previously.6,8 Mononuclear BMCs were collected from femurs and tibias of male GFP-transgenic rats, purified, and characterized as previously described.6

Generation of Post-MI Chronic Heart Failure and Cell Injection
Female wild-type SD rats (150 to 200 g. Harlan UK) underwent permanent left coronary artery (LCA) ligation as described before.6,9 Three weeks after LCA ligation, the animals were randomly assigned to three groups and either cells (5×10^6 SMBs) or 10×10^6 mononuclear BMCs in 100 μL PBS or 100 μL PBS were injected into two sites of the peri-infarct area using a 31-gauge needle through rethoracotomy.6 Although there was a concern that using SD rats for the donor/recipient of cell transplantation might cause immune response, results using athymic nude rats as recipients for the SD GFP+ cells (including LVEF improvement, changes in the cardiac parameters such as HR, LVd/Ds and peak E/A, histological inflammation and graft survival - data not shown) were very similar to those described here.

Assessment of Cardiac Function, Spontaneous Arrhythmia, and ECG Wave Pattern
Left ventricular ejection fraction (LVEF) was assessed by 2-dimensional mode echocardiography, and diastolic (LVd/Ds) and systolic (LVds) LV dimensions were measured with M-mode (Sequoia 512 and 15-MHz probe, Siemens Medical, n=10 for each group at each time point).6 Spontaneous arrhythmia occurrence was assessed by radio-telemetry (Data Sciences International) as described previously.6 The ECG was continuously monitored until day 28 after injection (n=7 in the PBS and BMC groups, n=8 in the SMB group). The hourly number of ventricular premature contractions (VPC) was calculated as an average over 24-hour recording.

Histological Analysis
Hearts were collected, perfuse-fixed with 4% paraformaldehyde, and frozen (n=3 in each group at each time point). Fifteen-μm cryosections were labeled with anti-GFP (1:1000 dilution, Molecular Probes), anti-CD45 (1:250 dilution, BD Pharmingen), and anti-Cx43 (1:500 dilution, Chemicon) antibodies. Fluorophore-conjugated secondary antibodies (Molecular Probes) were applied and nuclei stained with DAPI (Sigma) where required. Images were recorded by confocal microscopy (LSM510, Zeiss). For Cx43, all images were recorded using the same settings and the pixel intensities were quantified using ImageJ software.

Western Blotting
Powdered samples or cell monolayers were solubilized in 20% SDS by sonication and 10 μg total protein was separated electrophoretically, transferred to PVDF membrane, and probed using monoclonal anti-Cx43 (1 in 500, Chemicon) or antisarcosarcomactin actin (1 in 250, Daco Cytomation) antibodies. HRP-conjugated secondary antibodies were applied (1 in 1000, Chemicon) and bands revealed by ECL (GEHealthcare UK Ltd) and analyzed using ImageJ software. Cx43 levels were normalized to the sarcomeric actin levels for the same samples and then to the values obtained for the control heart samples which were run on all blots.

Quantitative Analysis of Graft Survival by Real-Time PCR for Sry
Male grafted cells in the female LV were quantified to define graft survival, by using real-time PCR (ABI PRISM 7700 and TaqMan chemistry) for the Y-chromosome specific Sry gene in DNA extracted from the powdered LV samples.6 Sry levels were normalized using the autosomal single copy gene osteopontin.11 A standard curve was prepared from DNA extracted from a mixture of 5×10^6 male SMBs or 10×10^6 male BMCs and female LV walls 21 days after LCA ligation from which the number of surviving cells was estimated.

Real Time RT-PCR
Total RNA purified from the powdered LV samples was reverse transcribed with random priming (Applied Biosystems). The resulting cDNA was used for real-time PCR for IL-1β and IL-6. GAPDH levels were simultaneously measured for normalization of RNA loading. Data obtained were analyzed with the Ct method using the Sequence Detection System. Levels were normalized to that observed in normal myocardium which was assigned a value of 1.

Collection and Culture of Neonatal Cardiomyocytes
Two-day-old neonatal SD rat hearts (atria removed) were finely chopped and digested with collagenase type II (Worthington) and penicillin (Sigma) at 37°C over a period of 110 minutes.12 Dissociated cells were collected at intervals, pooled, and fibroblasts removed by 45-minute preplating. Cardiomyocytes, seeded at 0.5×10^6 cells per well in gelatin-coated 12-well plates, were cultured in DMEM with 10% 199 medium, 10% horse serum, 5% FBS with and without IL-1β (20 ng/mL; BioSource).  

Scrape Loading
Confluent monolayers of neonatal cardiomyocytes were washed with PBS and covered with Lucifer yellow (Sigma, 0.2% in PBS). The monolayers were then scored/scraped with a scalpel, incubated for 5 minutes at 37°C, washed with PBS, and observed immediately by fluorescence microscopy and images recorded by digital camera (Nikon).13

Statistical Analysis
All values are expressed as mean±SEM. Data from LVEF, graft survival, frequency of spontaneous VPC, expression of Cx43, IL-1β, and IL-6 were all compared using 2-way ANOVA followed by the
Figure 1. Functional improvement and graft observation after cell therapy. A, Serial analysis of 2-D mode echocardiography showed a significantly greater LVEF in the two cell-treated groups compared to the PBS control. \( P < 0.05 \) vs PBS group, \( n = 10 \) in each group. B, Quantitative PCR for Y-chromosome-specific Sry gene revealed that the percentage survival of both cell types within the LV myocardium was poor, \( n = 5 \) in each group. C, Table showing the changes in the left ventricular dimensions in the three groups. \( P < 0.05 \) vs the PBS group. D through I, Immunolabeling for GFP demonstrated that grafted GFP\(^*\) cells remained as discreet cell-clusters, isolated from the native myocardium after BMC injection (D: day 7, E: day 28) and SMBs (F & H: day 7, G & I: day 28). Graft size decreased from 7 days to 28 days after injection but accurate quantification of the number of surviving cells was not possible by histology. GFP\(^*\) BMC and SMB grafts were surrounded by CD45\(^*\) inflammatory cells (D, E and F, G respectively). Cx43-gap junctions were absent between grafted GFP\(^*\) cells and native cardiomyocytes (H and I). Green=GFP and blue=nuclei (DAPI). Bar marker=50 \( \mu \)m.
Bonferroni post test for individual significant differences between the different groups at each time point. ECG parameters such as duration of QRS complex and frequency of induced VT were compared using the \( \chi^2 \) test. VPC number before and after isoproterenol administration were compared using the paired \( t \) test (1-tailed). \( P < 0.05 \) was considered to be statistically significant.

**Statement of Responsibility**
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**Functional Improvement With Low Graft Survival**
At 28 days after either SMB or BMC direct intramyocardial injection, LVEF was significantly and similarly improved, compared to before cell injection and PBS control (Figure 1A). The LVDs in the PBS group was enlarged after 28 days (Figure 1C), but this enlargement was not observed in the SMB or BCM groups. The LVDd was similarly enlarged in all groups after 28 days. Quantitative analysis using real-time PCR for the Y-chromosome–specific Sry gene demonstrated that graft survival was extremely poor after either SMB or BMC injection (Figure 1B). Only less than 1% of the total injected cells remained in the heart at day 28. Immunolabeling for GFP demonstrated the presence of surviving donor-derived GFP-expressing cells within the host myocardium at day 7 and 28 after either SMB or BMC injection (Figure 1D through 1I). At day 7 after injection of either cell type, islet-like GFP-expressing cell-clusters were found in the peri-infarct area (Figure 1D, 1F, 1H). In contrast, at day 28, the size of the GFP-expressing grafts had largely decreased (Figure 1E, 1G, 1I). It is noted that all these GFP-expressing cells appeared to be isolated from the native myocardium with inflammatory, CD45 cells (Figure 1D through 1G). More CD45 cells were apparent after SMB injections (Figure 1F) than after BMC injection (Figure 1D). Cx43-gap junctions were never observed between graft-derived SMBs and host cardiomyocytes at either day after injection (Figure 1H and 1I). In addition, GFP cardiomycocyte-like cells were not observed.

**ECG Changes and Arrhythmia Occurrence After SMB Transplantation**
Using radio telemetry, all animals showed typical ECG wave patterns for chronic myocardial infarction such as abnormal Q wave and ST elevation (data not shown). However, the QRS duration was significantly elongated at 28 days after SMB transplantation compared to those after BMC and PBS injections (Figure 2A). In addition, the SMB group demonstrated an increased incidence, albeit low, of spontaneous VPC at 28 days compared to other groups and before cell therapy (Figure 2B). As the aim of the study was to examine the late-phase arrhythmogenesis, data for the early stages after cell injection are not shown. Spontaneous occurrence of VT was not observed at 28 days in any group. For testing the importance of such VPC occurrence in the late stage after SMB injection, isolated sample-hearts were perfused and stimulated by isoproterenol-administration. It was revealed that only SMB-injected hearts developed a marked increase in VPC frequency and, furthermore, VT was provoked in more than 80% (5 of 6) of SMB-injected hearts (Figure 2C).

**Global Reduction in Cx43 After SMB Transplantation**
Immunolabeling demonstrated that the main ventricular gap junction protein, Cx43, was dramatically and globally reduced within the host myocardium after SMB transplantation compared to PBS and BMC groups. Western blotting provided results consistent with the immunolabeling quantification (Figure 3G through 3I). Quantitative RT-PCR revealed a remarkable, though statistically not significant, upregulation of IL-1\( \beta \) and IL-6 in the myocardium after SMB transplantation compared to PBS and BMC injections (Figure 4A and 4B). Upregulation of these proinflammatory cytokines continued at least for 7 days. To examine whether there was a link between the cytokine upregulation and Cx43 downregulation, we examined the effect IL-1\( \beta \) on cultured neonatal cardiomyocytes. Western blotting demonstrated that exposure of cultured cardiomyocytes to IL-1\( \beta \) decreased Cx43 expression after 24
and 48 hours (Figure 4C). Further, scrape-loading showed that functional intercellular communication via gap junctions between cardiomyocytes was clearly reduced by IL-1β administration (Figure 4D and 4E), but not by IL-6 administration (Figure 4F).

**Discussion**

The present study demonstrated that intramyocardial injection of SMBs and BMCs similarly improved cardiac function of postinfarction chronically failing hearts. The graft survival was poor, and cardiomyogenic differentiation or fusion with cardiomyocytes was not observed after injection of either cell type. In addition, gap junctions between grafts and native cardiomyocytes were absent. These findings in our rat model are an accurate reproduction of the observations in previous clinical/experimental studies.²,¹¹,¹⁴-¹⁶ In this appropriate model, which is free from antiarrhythmia drugs, we have shown that injection of SMBs, but not BMCs, induced spontaneous VPC with latent VT, which were also associated with elongated QRS duration, in the late phase after cell therapy. Of note, myocardial Cx43 expression was globally and persistently reduced only after SMB-injected hearts. These data provided principal proof of a new concept that adverse indirect effects specific to the donor cell-type cause
global reduction of gap junction formation within the host failing myocardium, contributing to development of the late-phase arrhythmogenicity. In addition, inflammatory cytokines including IL-1β were suggested to play a role in this pathological event.

We demonstrated the occurrence of late-phase arrhythmias after SMB transplantation which was not apparent after BMC transplantation. It has been suggested that SMBs have or acquire arrhythmogenic potential and could be a focus for ventricular arrhythmias after injection. Leobon et al have demonstrated that myotubes differentiated from SMBs are able to evoke action potentials intracellularly and also that electric stimulation of myotubes evokes a slow voltage-dependent discharge with superimposed bursts of action potentials. Additionaly, Abraham et al reported that direct interaction between SMBs and cardiomyocytes can provide electrophysiological milieu for reentrant arrhythmia. In an acute infarct mouse model, injection of SMBs did not prevent induced VT and significantly increased the incidence of sustained arrhythmias after in vivo pacing. Injection of Cx43-expressing SMBs, however, conferred protection against the induced VT, demonstrating the importance of electric coupling between host and donor cells. Apart from the direct arrhythmogenic potential of SMB-derived grafts, we provide evidence that the native cardiomyocytes in the chronically failing heart, particularly their Cx43-gap junctions, are widely and adversely modulated by indirect effects of the SMB transplantation. Such a global reduction in myocardial Cx43 would explain the elongated QRS duration after SMB injection observed in this study. The pathological change in gap junction formation and resulting altered conduction velocity in the host myocardium would contribute to the development of ventricular arrhythmias by amplifying electrophysiological heterogeneity, promoting generation of reentry or increasing vulnerability to arrhythmogenic stimuli. Further investigation of the changes in conduction using techniques such as optical mapping would provide more accurate spatial data.

It has recently been reported that indirect/paracrine effects, instead of cardiomyogenic differentiation, may play an important role in cell therapy–induced therapeutic benefits. Such benefits include enhanced neovascular formation, decreased fibrosis, and reduced cardiomyocyte hypertrophy and apoptosis. In contrast, this study proposed a unique concept of an “adverse” indirect effect after SMB injection: reduction of gap junctions between native cardiomyocytes. Although the molecular pathway for this reduction in Cx43 remains uncertain, we suggest that persistent upregulation of myocardial IL-1β may be a key player. Our study demonstrated that SMB injection induced a markedly high level of IL-1β for at least 1 week compared to BMC injection. There are several possible sources of IL-1β after SMB injection into the heart: native cells including accumulated inflammatory cells and cardiomyocytes, grafted SMB-derived cells, or a combination of these. Consistent with the previous reports using astrocytes and hepatocytes which demonstrated a downregulation of gap junction formation, our in vitro experiments showed that exposure to IL-1β caused both a decrease in Cx43 expression

Figure 4. Proinflammatory cytokines and gap junction formation. A and B, Real-time RT-PCR analysis showed marked upregulation of IL-1β (A) and IL-6 (B) in the heart tissue from the SMB-treated group. The levels are normalized to that found in normal myocardium which was set to a value of 1. n = 5 in each group. C, Western blotting showed that rat neonatal cardiomyocytes cultured in the presence of 20 ng/mL IL-1β downregulated Cx43 expression at 24 and 48 hours. n = 4 in each group. D through F, Gap junction communication, as assessed by scrape-loading dye transfer, was reduced in neonatal cardiomyocyte monolayers exposed to IL-1β (E) compared to control (D) or IL-6-treated cultures (F). Bar marker = 200 μm. Representative pictures from 3 experiments.

A

B

C

D

E

F
and in gap junctional communication in cultured cardiomyocytes. It is reported that Cx43 is downregulated in several types of heart failure including postinfarction ischemic cardiomyopathy. SMB injection and associated upregulation of proinflammatory cytokines might potentiate or accelerate such a pathological process. However, there will be other molecules or intercellular processes affected by SMB transplantation which could also play a role in the Cx43 down-regulation and may also cause additional proarrhythmogenic alterations such as changes to ion channel functioning. Further investigation to determine the responsive molecular mechanism is warranted.

In summary, although cell therapy is capable of improving function of the postinfarction chronically failing heart, there is late-phase arrhythmogenicity specific to donor cell-type. Global downregulation of Cx43 in the host myocardium was indicated to be an important factor underlying such arrhythmogenicity after SMB transplantation. Careful observation and appropriate prevention/treatment of such arrhythmogenicity will be required in future clinical applications of cell therapy especially using SMBS.

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Disclosures
None.

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