Direct Intramyocardial But Not Intracoronary Injection of Bone Marrow Cells Induces Ventricular Arrhythmias in a Rat Chronic Ischemic Heart Failure Model

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Background—Therapeutic efficacy of bone marrow (BM) cell injection for treating ischemic chronic heart failure has not been established. In addition, experimental data are lacking on arrhythmia occurrence after BM cell injection. We hypothesized that therapeutic efficacy and arrhythmia occurrence induced by BM cell injection may be affected by the cell delivery route.

Methods and Results—Three weeks after left coronary artery ligation, wild-type female rats were injected with $1 \times 10^7$ mononuclear BM cells derived from green fluorescent protein–transgenic male rats through either a direct intramyocardial or a retrograde intracoronary route. Both intramyocardial and intracoronary injection of BM cells demonstrated similar improvement in left ventricular ejection fraction measured by echocardiography and a similar graft size analyzed by real-time polymerase chain reaction for the Y chromosome–specific $Sry$ gene. Noticeably, intramyocardial injection of BM cells induced frequent ventricular premature contractions ($108 \pm 73$ per hour at 7 days after BM cell injection), including multiform, consecutive ventricular premature contractions and ventricular tachycardia for the initial 14 days; intracoronary injection of BM cells and intramyocardial injection of phosphate-buffered saline rarely induced arrhythmias. Immunohistochemistry demonstrated that intramyocardial BM cell injection formed distinct cell clusters containing donor-derived cells and accumulated host-derived inflammatory cells in the infarct border zone, whereas intracoronary BM cell injection provided more homogeneous donor cell dissemination with less inflammation and without disrupting the native myocardial structure.

Conclusions—BM cell injection is able to improve cardiac function in ischemic chronic heart failure but has a risk of arrhythmia occurrence when the intramyocardial route is used. Such arrhythmias may be prevented by using the intracoronary route. (Circulation. 2007;115:2254-2261.)

Key Words: arrhythmia ■ cell therapy ■ heart failure

Recent research has shown that injection of bone marrow (BM)–derived stem cells improves function and perfusion of the heart with acute myocardial infarction (MI) in animals and humans. However, therapeutic efficacy of this approach for treating post-MI chronic heart failure (HF) has not been fully established. In addition, arrhythmia occurrence, which has been identified as a critical complication of skeletal myoblast injection, has not been fully studied in the case of BM cell injection. Previous clinical studies reported rare incidences of arrhythmia after intramyocardial and antegrade intracoronary injection of BM cells; however, patients in these studies were prescribed antiarrhythmic drugs such as $\beta$-blockers, which could mask arrhythmogenicity of the treatment. In addition, arrhythmia occurrence in these patients might not be adequately examined by appropriate continuous monitoring. Elucidating these issues by appropriate laboratory investigations is now an urgent requisite for the further progress of clinical application of the treatment.

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We hypothesized that therapeutic efficacy and arrhythmia occurrence induced by cell injection into the post-MI heart may be affected by the cell delivery route. The direct intramyocardial route, either by an endocardial or epicardial approach, can deliver cells selectively into target areas; however, this method causes mechanical injury and subsequent acute inflammation. Such adverse processes could limit graft survival and cause myocardial damage. In addition, cells grafted via the intramyocardial route tend to form isletlike cell clusters that are isolated from the host myocardium. Such local heterogeneity in the myocardium is considered a source of arrhythmias. In contrast, the intracoronary route can deliver donor cells more homoge-
neously with less mechanical damage and inflammation in the myocardium.17 This ability may allow for enhanced therapeutic effects and a reduced risk of arrhythmia.

In the present study, we investigated the therapeutic benefits and arrhythmia occurrence after BM cell injection for treating post-MI chronic HF by comparing intramyocardial and intracoronary injection.

Methods
All studies were performed with the 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 surgical procedures and evaluations, including assessment of ventricular function, were carried out in a blinded manner.

Donor Cell Preparation
Mononuclear BM cells were collected from both femurs and tibias of green fluorescent protein (GFP)-transgenic male Sprague-Dawley rats18 (n=13; 200 to 250 g; Rat Resource and Research Center, Columbia, Mo) and purified by Percoll gradient centrifugation (Amersham Biosciences, Piscataway, NJ) as previously described. Flows cytometry analysis (FACSARia, BD Biosciences, Erembodegem, Belgium) using monoclonal anti-CD34 (Santa Cruz Biotechnology, Santa Cruz, Calif) and anti-CD45 (BD PharMingen, Oxford, UK) antibodies showed that 4.6±1.7% of the collected BM mononuclear cells were positive for CD34 and 75.5±4.3% were positive for CD45.

Generation of Chronic Ischemic HF
Wild-type Sprague-Dawley female rats (n=150; 150 to 200 g; Harlan Sprague Dawley, Indianapolis, Ind) underwent left thoracotomy under 1.5% isoflurane inhalation and mechanical ventilation using a volume-controlled ventilator (Harvard Apparatus, Edenbridge, UK). The left coronary artery (LCA) was permanently ligated with 6-0 polypropylene (BearMedic, Chiba, Japan) at the level of the bottom edge of the left atrial appendage.17 Ischemia was confirmed by a change in the epicardial color and dyskinesis. The rats were returned to the cage for recovery after chest and skin closure and extubation.

Assessment of Cardiac Function
Cardiac function and dimensions of all the surviving rats were assessed at 20 days after LCA ligation with echocardiography using Sequoia 512 and 15-MHz probe (Siemens Medical, Berlin, Germany) under 1.5% isoflurane inhalation via a nose cone. Left ventricular ejection fraction (LVEF) was calculated from the data obtained with 2-dimensional tracing. LV diastolic (LVDd) and systolic (LVDs) dimensions and heart rate (HR) were measured with M-mode echocardiography. Transmitral peak E/A flow ratio was determined by spectral Doppler tracers. Changes in cardiac parameters were assessed at 3, 7, 14, 28, and 84 days after cell injection (n=9 in each group). Therapeutic efficacy was evaluated primarily by a positive change in LVEF after cell injection.

Arrhythmia Assessment by Telemetry
A transmitter for the radiotelemetry (Data Sciences International, St Paul, Minn) was implanted in the peritoneal cavity 1 day before cell injection for continuous ECG monitoring (n=7 in each group). The electrodes were implanted in the right shoulder and left axilla. The ECG of these rats was monitored continuously for 1 day before until 7 days after cell injection, followed by 24 hours of continuous monitoring at 14, 28, 42, 56, and 84 days. The hourly number of ventricular premature contractions (VPCs) was calculated as the average number of the events observed in a 24-hour recording with ECG analysis software (DSI Dataquest A.R.T. Analysis, Data Sciences International). The frequency of multiform, consecutive VPCs and ventricular tachycardia (VT) also was counted throughout the periods studied.

Cell Injection Into the Heart
The rats that met the criteria (LVEF <40%) 1 day before cell injection were assigned to cell injection. At 21 days after LCA ligation, under 1.5% isoflurane inhalation and mechanical ventilation, 1×10^7 mononuclear BM cells were injected via either direct intramyocardial (BMC-IM group; n=34) or retrograde intracoronary (BMC-IC group; n=35) route through rethoracotomy. In the former method, BM cells suspended with 100 μL phosphate-buffered saline (PBS) were injected into 2 sites of the infarct border zone: the anterior-lateral and inferior-posterior walls. In the latter method, the same number of BM cells in 500 μL PBS was injected into the left cardiac vein, which drains the entire LV free wall, through a purse-string suture (7-0 polypropylene, BearMedic) placed on left superior vena cava as recently described. The stem of the cardiac vein was snared for 30 seconds after injection to prevent flushing the injected cells into the vena cava. As controls, the same volume of PBS without cells was injected via either route (PBS-IM group, n=26; and PBS-IC group, n=28). The rats were returned to the cage for recovery after chest and skin closure and extubation.

Survival of Grafted Donor Cells
The presence of grafted male cells in the female heart was quantitatively assessed by real-time polymerase chain reaction for the Y chromosome–specific Sry gene (ABI PRISM 7700 and TaqMan Chemistry; Applied Biosystems, Foster City, Calif). At 3, 7, 28, and 84 days after cell injection, the LV wall was collected (n=4 to 6 at each time point in the BMC-IM and BMC-IC groups). Genomic DNA was extracted from the sample and used for Sry detection. The signals obtained were normalized for the amount of DNA used by the autosomal single-copy gene osteopontin (Opn) as an internal standard.12 Primers used were as follows: Sry: forward, 5'-GCCGAGAGAGGCACAAGGTG-3'; reverse, 5'-TCCCGAGCTTGTGCTGATC-3'; and probe, 5'-TCAACAGAATCCCAGCA; Opn: forward, 5'-CACCTGCCCCACAGT-3'; reverse, 5'-CTCTAGCTCGAGCCAAGCT-3'; and probe, 5'-TGTTCCCTGACGGCGAGGTGA-3'. Female LV at 21 days after LCA ligation was mixed with male BM cells (1×10^7), and its serial dilution series was used for generating a standard curve (n=3).

Histological Analysis
The rats were killed at 3, 7, and 28 days after cell injection for histological analysis (n=4 to 5 at each time point in each group). The hearts were perfused with 4% paraformaldehyde immediately after collection and frozen in liquid nitrogen. Cryosections (10 μm) were labeled with a polyclonal GFP-specific primary antibody (Molecular Probes, Carlsbad, Calif; 1:5000 dilution),13 which was visualized with horseradish peroxidase–based Envision kit (Dako, Glostrup, Denmark) according to the manufacturer’s instructions. The samples were counterstained with hematoxylin.

Statistical Analysis
All values are expressed as mean±SEM. Statistical comparison of LVEF, HR, LVDd/Ds, peak E/A, and number of VPCs was performed with 1-way ANOVA, followed by Bonferroni’s test for individual significant difference. A value of P<0.05 was considered statistically significant.

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
Mortality and Arrhythmia Occurrence
Mortality after LCA ligation before cell injection was 14% (21 of 150). Among the 129 surviving rats, 6 (4.7%) were excluded from the study before cell injection because their
LVEF was >40%. Mortality after cell injection was similarly low in all groups: 5.9% (2 of 34) in the BMC-IM, 5.7% (2 of 35) in the BMC-IC, 3.8% (1 of 26) in the PBS-IM, and 7.1% (2 of 28) in the PBS-IC group. All mortalities occurred during the procedure or immediately after, presumably as a result of surgical stresses such as bleeding.

Continuous ECG monitoring did not detect frequent (>1 per hour) VPCs in any rats 1 day before cell injection, whereas after cell injection, temporary but frequent VPCs were observed in the BMC-IM group. In this group, a large number of VPCs were detected as early as 1 day (15±5 per hour) after cell injection. The number of VPCs peaked at 7 days (108±73 per hour), and the frequent VPCs largely disappeared by 28 days after BM cell injection (Figure 1A). All other groups showed only a limited number of VPCs (<1 per hour) throughout the periods studied. More than 70% of rats in the BMC-IM group showed multiform (Figure 1B) or consecutive VPCs between 1 and 7 days after BM cell injection (Table 1). The proportion of rats showing VT (Figure 1C) was 14% at 1 day, 43% at 7 days, 0% at 28 days, and 0% at 84 days in the BMC-IM group. All other groups did not show VT throughout the periods studied.

**Improved Cardiac Function After BM Cell Injection**

Baseline values (intact normal rats; n=11) of LVEF, HR, LV Dd/LVs, and peak E/A were 74.1±1.4%, 365±15 bpm, 6.7±0.3/3.7±0.2 mm, and 1.9±0.0, respectively. At 20 days after LCA ligation (1 day before cell injection; n=123), echocardiography showed a consistently reduced LVEF (33.6±0.4%: P<0.001) and E/A (1.2±0.1; P<0.001) and an enlarged LV Dd/LVs (8.9±0.1/7.6±0.2 mm; P<0.001) compared with the baseline values. HR (370±8 bpm) did not show a significant change.

After cell injection, both the BMC-IM and BMC-IC groups demonstrated a similar degree and time course of improvement in LVEF (Figure 2). LVEF significantly increased by 7 days after cell injection but not at 3 days in the BMC-IM (49.1±1.5%) and BMC-IC (49.0±1.4%) groups compared with before cell injection (34.6±1.1% and 33.5±0.9%, respectively). Significantly improved LVEF remained until 84 days in both groups. LVEF in the BMC-IM and BMC-IC

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<th>TABLE 1. Percent of Sample Showing ≥1 Multiform or Consecutive VPCs per Day</th>
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<td>Days After Cell Injection</td>
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Multi indicates multiform VPCs; Consec, consecutive VPCs. n=7 each group.
groups also was significantly higher than that in the PBS-IM and PBS-IC groups, respectively, throughout the periods between 7 and 84 days after injection. The other cardiac parameters also indicated improvement in cardiac performance after BM cell injection via either route (Table 2). The enlarged LVDs, reduced peak E/A, and increased HR observed in the PBS-IM and PBS-IC groups at 28 days compared with before cell injection did not occur in the BMC-IM and BMC-IC groups.

**Survival of Grafted Donor Cell**
Survival of male donor cells in the female host failing heart was quantitatively analyzed by real-time polymerase chain reaction for Sry. The BMC-IM and BMC-IC groups showed a similar small number of male cells present throughout the time points studied (Figure 3). At 3 days after cell injection, a similar small number of donor cells remained in the host LV throughout the time points studied. At 7 days after cell injection, a very small number of male cells were detected in the BMC-IM and BMC-IC groups, respectively. The number of surviving donor cells further decreased to 0.07 ± 0.03% by 7 days, respectively. The graft survival was then stable until 84 days after cell injection in both groups.

**Distribution of Grafted Cells in the Host Myocardium**
Grafted cells were detected by immunohistochemistry for GFP. The BMC-IM group showed GFP-positive, discrete cell clusters in the border zone surrounding the infarcts at 3 days after cell injection (Figure 4A). These cell clusters were composed of GFP-positive grafted cells and GFP-negative host-derived cells (Figure 4B). The BMC-IM group at 7 days showed a persistent presence of the cell clusters but now composed of a reduced number of GFP-positive cells and many GFP-negative cells (Figure 4C). Most of the GFP-positive cells at 3 and 7 days were hematopoietic-like round cells, whereas some cells had a more elongated appearance. Most GFP-negative cells found in the cell clusters were identified as accumulated inflammatory cells, including polymorphonuclear leukocytes and lymphocytes having large round nuclei.

In contrast, the BMC-IC group showed more homogeneous cell dissemination into both infarct and noninfarct areas. The structure of adjacent myocardium in the BMC-IC group was better preserved compared with the BMC-IM group (Figure 4D). In the BMC-IC group at 3 and 7 days, inflammatory cells were rarely detected around the disseminated GFP-positive grafted cells (Figure 4E and F). Despite thorough histological examinations, we did not detect any GFP-positive cells entrapped in the vasculature, including cardiac veins, venules, capillaries, arterioles, or arteries, in the BMC-IC group. At 28 days after BM cell injection, a very small number of GFP-positive cells were detected in the infarct and border zones of both the BMC-IM and BMC-IC groups, associated with little detectable inflammatory response. Clear evidence of differentiation of GFP-positive cells into mature cardiac phenotype was not found in any samples of either the BMC-IM or BMC-IC group (data not shown).

**Discussion**
The present article provides experimental evidence that direct intramyocardial injection of BM cells can induce a serious high frequency of and critical types of ventricular arrhythmias for the initial 14 days after injection in post-MI chronic HF. Such arrhythmia did not occur after retrograde intracoronary injection of BM cells, despite the fact that the 2 injection methods resulted in similar therapeutic benefits and similar graft sizes. These data have important implications in developing the optimal protocol for cell therapy using BM cells.

Arrhythmogenicity of intramyocardial injection of BM cells has not been discussed extensively, probably because previous clinical trials have reported negative results. However, the patients studied in these trials were medically treated with drugs having an antiarrhythmic effect such as...
β-blocker, which would have masked the arrhythmogenicity. In addition, for detecting arrhythmias, these studies monitored patients only for <2 days after cell injection9 and/or at 1 month or later.5,8 However, these protocols for monitoring ECG might not be appropriate. Our results demonstrated that arrhythmia occurrence peaked at day 7 after intramyocardial injection of BM cells, when we observed the most frequent VPCs (108 per hour; classified into Lown 2), with multiform/consecutive VPCs (Lown 3/4a) in 71% of treated rats and VT (Lown 4b) in 43%. This original information suggests that approximately day 7 after treatment is extremely important in evaluating the arrhythmogenicity of BM cell injection, therefore proposing that future clinical studies should include intensive monitoring around this time point to further clarify this issue in patients.

Our findings suggest that the ventricular arrhythmias observed in the early phase after intramyocardial BM cell injection might be due to the generation of cell clusters in the border-zone myocardium. These isolated cell clusters, composed of donor-derived cells and accumulated inflammatory cells, would produce an adverse physical effect (severe local heterogeneity within the myocardium), disturbing the myocardial consistency and the conductance properties.15,16 In addition, the accumulated inflammatory cells within the clusters are likely to cause an adverse biochemical effect on surrounding cardiomyocytes by inducing local myocardial upregulation of various proinflammatory cytokines such as interleukin-1β and tumor necrosis factor-α as shown previously.13,14 Consequently, cardiomyocyte apoptosis, modulation of gap junction formation, and/or deterioration of electrical stability may be amplified particularly in the border zone where native cardiomyocytes suffer post-MI adverse remodeling processes, eventually causing ventricular arrhythmias.24 This speculation is supported by the findings in our study that homogeneously disseminated BM cells with little inflammation by retrograde intracoronary injection or mild-degree inflammation by intramyocardial injection of PBS only did not cause significant ventricular arrhythmias. VPC frequency appeared to be higher at day 7 after treatment compared with day 3 (although not significant) in our study. The precise reason for this is unclear; however, persistent stress, including continuous exposure to proinflammatory cytokines, might progressively cause an adverse effect on failing cardiomyocytes in the infarct border area, increasing the frequency of VPCs toward day 7 after injection.

Another possible cause for ventricular arrhythmias after BM cell injection is the direct adverse effect of the grafted cells such as inappropriate cardiomyogenic differentiation, fusion with native cardiomyocytes, and/or formation of inadequate intercellular connections with surrounding native cardiomyocytes.25,26 It has been reported that BM cell–derived cardiomyocytes trigger arrhythmogenic sparks in vitro.27,28 However, our results provided several pieces of negative evidence for this view. First, arrhythmias observed after intramyocardial injection of BM cells were largely absent by retrograde intracoronary injection, despite a similar number
of surviving grafted cells. If direct adverse effects of grafted cells were the main cause of the arrhythmia, frequent arrhythmias should have been similarly observed after intracoronary BM cell injection. Second, frequent VPCs were found as early as 1 day, with the peak frequency at 7 days, after intramyocardial BM cell injection. Such a time course seems too short for grafted BM cells to undergo cardiomyogenic differentiation, which usually takes several weeks in vitro. Third, the frequency of such transdifferentiation events is reported to be extremely low. In fact, we could not detect any clear evidence indicating cardiomyogenic differentiation of grafted cells despite thorough histological examinations. In addition, ectopic stimuli derived from such a small number of differentiated cells, if any, might be diluted by much larger number of surrounding cardiomyocytes and would not lead to ventricular arrhythmias.

Our study demonstrated the principal concept that intramyocardial BM cell injection could induce serious ventricular arrhythmias in a rat chronic ischemic HF model. However, because several differences exist in morphological and electrophysiological properties between rat and human myocardium, further large-animal or clinical studies would be useful to verify our findings in a clinical setting. It also would be interesting to investigate whether the modulation of injection protocol, such as injections of a smaller volume into a larger number of sites, could reduce arrhythmia occurrence after intramyocardial injection. It has been reported that such multiple injections of a smaller volume improve cardiac function more efficiently compared with a single injection of a large volume. Furthermore, electric mapping approaches, available in human and large animals, may be useful for identifying the high-risk areas for cell injection–induced arrhythmia, possibly reducing the risk of arrhythmias.

Therapeutic efficacy of mononuclear BM cell injection for treating post-MI chronic HF was inconsistent among previous clinical studies. Perin et al., Beeres et al., and Strauer et al. observed a significant improvement in LVEF, whereas Fuchs et al., Kuethe et al., Tse et al., and Blatt et al. reported negative results. Although the reasons of such a discrepancy in LVEF improvement are not known, one may speculate that it results from the heterogeneous nature of patients assigned to the study, the small numbers of patients, the variable quality of BM cells, and the different injection methods, including the site for injection and the cell number injected. On the other hand, in this study, we have provided evidence that mononuclear BM cell injection by either route consistently improves both systolic (ie, LVEF) and diastolic (ie, peak E/A ratio) cardiac functions of post-MI chronic HF using an established rat model. Because BM cell injection, regardless of the cell delivery route, failed to suppress progression of ventricular dilatation (ie, LVDd), LVEF improvement after BM cell injection appeared to be largely dependent on reduced LVDs. It is therefore indicated that mononuclear BM cell injection via either route can be an effective therapy for treating post-MI chronic HF but is subject to patient selection and optimization of the protocol. Quantitative analysis of surviving donor-derived cells using real-time polymerase chain reaction showed that <0.1% of injected cells existed in the heart at day 7, whereas significant functional improvement was observed. Although the direct mechanical contribution of such a small number of surviving donor-derived cells is unlikely to be sufficient to increase global cardiac function, these cells could, by a paracrine effect, improve the contractility of a much larger number of surrounding failing cardiomyocytes. In addition, other paracrine effects mediated by the donor-derived cells such as increased neovascular formation would also be useful to enhance cardiac function.

Antegrade intracoronary injection has been shown to have a similar ability to disseminate cells into the myocardium. However, retrograde intracoronary injection is believed to have several possible advantages over antegrade intracoronary injection. Although the antegrade intracoronary route is commonly used in clinical trials, this method reportedly carries a risk of coronary embolism. This risk will be particularly critical during the treatment of post-MI chronic HF when coronary arteries often show diffuse narrowing at many sites with jeopardized collateral arteries. In contrast, the risk of coronary embolism will be largely obviated in retrograde intracoronary injection. Furthermore, cells injected via the retrograde intracoronary route may have a greater chance for transendothelial migration from the intravascular space into the myocardial interstitium. It is known that migration of inflammatory cells into the myocardial interstitium takes place at postcapillary venules rather than at capillaries or arterioles as a result of the endothelial-leukocyte interaction in which a distinct set of adhesion molecules plays a role. The retrograde intracoronary route is able to deliver cells directly to this unique part of the vasculature, resulting in enhanced transendothelial donor cell migration. Retrograde intracoronary injection is feasible in clinical settings by use of a balloon catheter; however, further investigation is needed to prove these advantages of retrograde intracoronary injection over antegrade intracoronary injection. Because a reproducible model for antegrade intracoronary cell injection has not been established in small animals, a large-animal experiment will be more suitable for this purpose.

In summary, we provide evidence that direct intramyocardial injection of BM cells induces temporary but serious ventricular arrhythmias in a post-MI chronic HF model. Such arrhythmias were largely absent when injected via the retrograde intracoronary route despite the similar therapeutic efficacy. These data suggest that the retrograde intracoronary route might be a safe, efficient route for BM cell injection for treating post-MI chronic HF.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

Cell therapy using bone marrow cells is now being studied as a treatment for postinfarction chronic heart failure in patients. Although previous clinical studies reported rare incidences of arrhythmia, the arrhythmia risk may have been masked by the common use of antiarrhythmia drugs. In the present study, we compared the effects of intramyocardial and retrograde intracoronary injection of bone marrow mononuclear cells on ventricular arrhythmias using an established rat model. Injection of bone marrow cells by either route improved both systolic and diastolic ventricular function but with similarly poor graft survival. Evidence for differentiation of donor cells into cardiomyocytes or vascular cells was very rare, suggesting that paracrine effects may be important for the impact of therapy on ventricular function. Intramyocardial cell injection induced frequent ventricular arrhythmias, including ventricular tachycardia, for 14 days after injection. Intramyocardial injection caused the formation of localized clusters of donor-derived cells and host-derived inflammatory cells within the border-zone myocardium. In contrast, intracoronary injection disseminated cells with little inflammation and rarely induced arrhythmias throughout the period. These results show that the method of cell delivery can influence the inflammatory response and arrhythmogenesis. The findings support trials of bone marrow cell transplantation for treating postinfarction heart failure but indicate that further study of arrhythmia risk, particularly in the early phase after intramyocardial injection, is warranted.
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