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Human embryonic stem cell-derived cardiomyocytes engraft but do not alter cardiac remodeling after chronic infarction in rats

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Abstract

Background—Previous studies indicated that, in an acute myocardial infarction model, human embryonic stem cell-derived cardiomyocytes (hESC-CM) injected with a pro-survival cocktail (PSC) can preserve contractile function. Because patients with established heart failure may also benefit from cell transplantation, we evaluated the physiological effects of hESC-CM transplanted into a chronic model of myocardial infarction.

Methods and Results—Intramyocardial injection of hESC-CM with PSC was performed in nude rats at 1 month following ischemia-reperfusion. The left ventricular function of hESC-CM injected rats was evaluated at 1, 2 and 3 months after the cell injection procedure and was compared to 3 control groups (rats injected with serum-free media, PSC-only, or non-cardiac human cells in PSC). Histology at 3 months revealed that human cardiomyocytes survive, develop increased sarcomere organization and are still proliferating. Despite successful engraftment, both echocardiography and MRI analyses showed no significant difference in left ventricular structure or function between these 4 groups at any time point of the study, suggesting that human cardiomyocytes do not affect cardiac remodeling in a rat model of chronic myocardial infarction.

Conclusion—When injected into a chronic infarct model, hESC-CM can engraft, survive and form grafts with striated cardiomyocytes at least as well as was previously observed in an acute myocardial infarction model. However, although hESC-CM transplantation can attenuate the progression of heart failure in an acute model, the same hESC-CM injection protocol is insufficient to restore heart function or to alter adverse remodeling of a chronic myocardial infarction model.

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Keywords

heart failure; myocardial infarction; ischemia; cell transplantation; stem cells

Introduction

The transplantation of derivatives of human embryonic stem cells has received considerable attention as a potential new therapeutic strategy to regenerate human myocardium after infarction. Once injected in myocardial infarction models, human embryonic stem cell-derived cardiomyocytes (hESC-CM) can survive and form grafts of maturing striated human cardiomyocytes that are stable up to several months after injection [1-3]. Grafting of hESC-CM exerts beneficial effects on heart function, *e.g.* preservation of fractional shortening and attenuation of the classic course of ventricular remodeling in a rat model of myocardial infarction [1,4,5]. In contrast, grafting of non-cardiac hESC derivatives did not show any beneficial effects in these studies [1,5,6].

Previous studies reported beneficial effects of hESC-CMs after grafting injected the cells in the acute or subacute myocardial injury period [1-3]. To date, however, no studies have explored the suitability of hESC-CMs to repair chronically infarcted myocardium. The ability to restore function in the setting of chronic heart failure would be highly desirable from a clinical standpoint, but it also presents a greater challenge than preventing heart failure progression. Cell survival after engraftment is one limiting factor of cell-based cardiac therapy. The chronically infarcted heart may be a particularly hostile environment for cell engraftment, *e.g.* fibrous scar tissue with low vascularization. It remains uncertain whether hESC-CM transplantation could exert a beneficial effect in the chronically infarcted heart that has already undergone adverse remodeling. The current study aimed to evaluate long-term effects of hESC-CM transplantation in a chronic model of myocardial infarction.

Methods

Myocardial infarction model and cell transplantation

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was approved by our institutional animal care and use committee. The myocardial infarction model and cell transplantation protocol has been described previously in several reports by our group [1,7,8]. In brief, athymic male Sprague Dawley rats (rnu-rnu, 240–300g, Harlan) were intubated under general anesthesia (ketamine/xylazine, 70–100 / 7–10 mg/kg; IP) and mechanically ventilated. Myocardial infarction was induced by 60 min of ischemia-reperfusion injury (I/R; ligation of the left anterior descending artery by 7-0 Prolene suture). Three weeks after I/R, animals underwent echocardiographic evaluation and animals with fractional shortening >40% were excluded to avoid studying animals with small infarcts. Qualified animals were randomly assigned to one of the 4 following groups: 1) hESC-CM group receiving hESC-derived cardiomyocytes suspended in pro-survival cocktail (PSC; see below), 2) non-cardiac group receiving non-cardiac hESC derivatives suspended in PSC, 3) PSC group receiving PSC only and 4) SFM group receiving intracardiac injections of serum-free media.

Preparation of hES cell-derived cardiomyocytes

All cells were differentiated from the female H7 human ES cell line at Geron Corporation as previously described [1]. Briefly, hESCs were maintained in the undifferentiated state on Matrigel-coated plates and fed daily with mouse embryonic fibroblast-conditioned medium,

supplemented with 8 ng/mL basic fibroblast growth factor (MEF-CM). Cardiac differentiation was induced in high density monolayers by replacing MEF-CM with RPMI-B27 medium (Invitrogen) supplemented with the following cytokines: 100 ng/mL human recombinant activin A (R&D Systems) for 24hrs, followed by 10 ng/mL human recombinant BMP4 (R&D Systems) for 4 days. The medium was then exchanged for RPMI-B27 without supplementary cytokines and cultures were fed every other day until beating foci, indicative of cardiomyocyte differentiation, appeared. For control experiments involving non-cardiac preparations, hESCs were differentiated under otherwise identical culture conditions, but activin A and BMP4 were omitted.

Differentiated hESCs (hESC-CMs and non-cardiac cells) were shipped overnight to the University of Washington 3 to 4 days prior to transplantation. To improve cell survival after grafting, 24 hrs before transplantation cultures were subjected to transient heat shock (30 min exposure to 42°C medium), followed by a return 37°C RPMI-B27 medium supplemented with IGF-1 (100 ng/mL) and cyclosporine A (0.2 mM) as described previously [1]. The following day, cells were enzymatically dispersed for implantation using Blendzyme IV (Roche, prepared at 0.56 U/mL in PBS) and DNase (Invitrogen, 60 U/mL) for 30 min. For hESC-CM transplantation, cell preparations were enriched for cardiomyocytes by separation over a discontinuous Percoll gradient [1]. Non-cardiac control cells were not Percoll-enriched, but processed equally otherwise, including heat shock, IGF-1 and cyclosporine A treatment. After centrifugation, hESC-CM or non cardiac cell preparations were suspended in pro-survival cocktail (PSC) consisting of 50% (vol/vol) growth factor–reduced Matrigel, supplemented with ZVAD (final concentration 100 µM, benzyloxycarbonyl-Val-Ala-Asp(O-methyl)-fluoromethyl ketone, Calbiochem), Bcl-XL BH4 (cell-permeant TAT peptide, 50 nM, Calbiochem), cyclosporine A (200 nM, Wako Pure Chemicals), IGF-1 (100 ng/mL, Peprotech) and pinacidil (50 µM, Sigma). The composition of the PSC and its efficiency to improve cell survival after intramyocardial injection is further detailed in Laflamme *et al.* [9].

Even after directed differentiation and Percoll gradient enrichment, hESC-CM preparations include both cardiomyocytes and non-cardiac cell types. We therefore determined the cardiac purity of each cell preparation. For this purpose, an aliquot of each cell batch was withdrawn from the final cell therapy product. A subset of these aliquots was cytospun and fixed for immunohistologic evaluation for β -myosin heavy chain and CD31 as cardiac and endothelial markers, respectively. Another subset was replated on gelatin coated plates for future immunostaining or patch clamp analysis. Because the division rate of cardiac cell and non-cardiac cell are different, those re-plated preparations were not use to assess the cardiac purity of the cell therapy product.

Patch clamp analysis

Spontaneous action potentials of hESC-CMs were recorded with a HEKA EPC-10 amplifier (HEKA, Lambrecht, Germany) in current-clamp mode at $36 \pm 1^\circ\text{C}$. Patch pipettes had a resistance of 2–4 M Ω when filled with the internal solution containing (in mM) 135 KCl, 5 Na, 2 creatine phosphate, 5 Mg-ATP and 10 HEPES, adjusted to pH 7.20 with KOH. Data were digitized at 10 Hz and filtered at 2.9 Hz through an LIH-1600 AD interface (HEKA) controlled by Patchmaster software (HEKA). Action potential parameters were analyzed using Patchmaster and/or IgorPro software (WaveMetrics, Inc. USA).

Cell transplantation

Cell or vehicle injections were performed 4 weeks after the initial ischemia-reperfusion infarct (1 week after baseline echocardiography; figure 1) as previously described [1]. In brief, the rats were anesthetized with ketamine/xylazine and subjected to a second

thoracotomy. A total of 10×10^6 cells suspended in 70 μ L of PSC or vehicle alone was injected using a 0.3 mL insulin syringe and a 29-gauge needle (Becton Dickinson). To ensure comparability between rats, all animals received 3 direct intramyocardial injections performed following the same pattern: 1 injection located at the center of the infarcted area and 2 injections at the border zone of the infarct. All rats received daily subcutaneous injections of cyclosporine A (0.75 mg/day, Wako Pure Chemicals) starting 1 day before engraftment and continuing for 7 days after engraftment.

Cardiac function evaluation

Cardiac function was evaluated by repeated echocardiography and MRI (described below) on lightly anesthetized animals (2% isoflurane; Novapulus). To ensure comparability between groups after the first echocardiography (baseline echocardiography), animals that met the inclusion criteria ($FS < 40\%$) were randomized between the 4 groups. All cardiac function evaluations were performed by an investigator blinded to the respective treatment.

Echocardiography was performed at 3 weeks after the I/R (1 week before engraftment) and at 1, 2 and 3 months after engraftment (Figure 1). The left ventricular end-diastolic dimension (LVEDD), the left ventricular end-systolic dimension (LVESD) and heart rate were measured by transthoracic echocardiography (GE Vivid 7) with a 10S (10 MHz) pediatric transducer. Fractional shortening was calculated by this equation: $FS = 100 \times (LVEDD - LVESD) / LVEDD$. To further evaluate left ventricular function, magnetic resonance imaging (MRI) was performed in a subset of animals at 1 and 3 months after cell transplantation. MRI was performed using a 3T Achieva Philips scanner and a custom-constructed 2-turn solenoid receive-only coil. Single-lead ECG was recorded from subcutaneous needle electrodes attached to animal's extremities and was used to trigger the MRI acquisitions using commercial software (Small Animal Monitoring and Gating System, SA Instrument Inc., Stony Brook, NY). Due to the heart rate limitations of the 3T human scanner (250 bpm) and high heart rate in rats (about 400 bpm) we triggered MR acquisitions on every other heart beat using SA Instrument software tools. A prospectively triggered, cartesian turbo-gradient echo cine (TFE CINE) sequence through the short-axis slices of the heart was used with a slice thickness of 1.5 mm without gap between slices, TR/TE 8.3/3.8 ms, field of view 70×49 mm, flip angle 30°, 2D acquisition matrix 232×163, two signal averages, phase interval 8.3 ms.

Epicardial and endocardial borders from magnetic resonance images were manually traced using ImageJ 1.34s software (NIH, USA) for determination of left ventricular chamber volumes at end systole and end diastole (ESV, EDV) and left ventricle mass (LV mass). Total LV volumes were calculated as the sum of all slice volumes. Stroke volume (SV) was calculated as EDV minus ESV; cardiac output (CO) as SV multiplied by heart rate (HR). The left ventricular ejection fraction (EF) was calculated by the equation $(EDV - ESV) / EDV \times 100\%$. Left ventricular wall thickness was measured in the center of the infarcted area, averaged in four short-axis slices, and in the opposite wall and the mid-septal wall of the same slices. Wall thickening was then calculated as the relative difference in LV wall thickness in end-systole and end-diastole and expressed as a percentage of end-diastolic thickness.

Histology, immunocytochemistry and in situ hybridization

One hour before sacrifice, rats received an intraperitoneal pulse of 5-bromodeoxyuridine (BrdU, 10 mg/mL solution prepared in PBS) to mark cells synthesizing DNA. Rats were sacrificed 1 or 3 months after cell injection using Beuthanasia (1.5–2 mL; *ip*, Schering-Plough). The hearts were harvested, transversely sectioned into 2-mm macro-sections from base to apex, the first slice being positioned at the site of the coronary occlusion. The

sections were immersion-fixed in methyl Carnoy's solution and paraffin-embedded for histology. Five-micrometer sections were then stained with hematoxylin/eosin. Picrosirius red staining was used to identify collagen. Grafts were identified by *in situ* hybridization using a human-specific pan-centromeric probe and immunohistochemistry using previously detailed methods [1]. Immunostaining was performed with antibodies directed against the *Beta*-myosin heavy chain isoform (β -MHC; clone A4.951, American Type Culture Collection), pan-cytokeratin cocktail (AE1/AE3, Dako), human specific endothelium (CD31/PECAM, Dako), erythroid cells (TER-119, BD Pharmingen) and pan-cadherin (rabbit polyclonal, Sigma). The host-derived endothelium was identified using a rat-specific endothelial antibody (rat endothelial cell antigen 1, Abcam). Proliferation was assessed by immunostaining for incorporated BrdU in combination with staining for β -MHC (sheep polyclonal biotinylated anti BrdU antibody, Abcam or peroxidase-conjugated mouse anti-BrdU monoclonal antibody, Roche for fluorescent or chromagen immunostaining respectively). For evaluation of the infarcted area or the graft size, digital photographs of section stained by picrosirius red or for β -Myosin Heavy were performed with a power Shot S51S digital camera (Canon Inc.) connected to a microscope SMZ1000 (Nikon). The area of the left ventricle and the graft cross-sectional area were quantified by a blinded reviewer using Adobe Photoshop software.

Confocal microscopy was performed on a Zeiss Confocal Microscope; model LSM510 Meta microscope.

Data Analyses

All data are expressed as mean $\pm$ SE. Statistical analyses were performed using Graph Pad Prism 4.0 software. MRI and echocardiography data were compared by 1-way and 2-way ANOVAs, using treatment and time as predictor variables. Values of $p < 0.05$ were considered statistically significant.

Results

The goal of this study was to determinate if a cell preparation that has been proven to be beneficial in an acute myocardial infarction model, would be effective when applied to a chronic myocardial infarction model. For this study, we chose to inject 10×10^6 cells from human embryonic stem cell derived cardiomyocytes preparation in 70 μ L of PSC, the same cell number and same vehicle that has been proven to have beneficial effect when injected into acute model of myocardial infarction. [1]. For comparison the entire left ventricle of the rat contains $\sim 20 \times 10^6$ cardiomyocytes [10].

Characteristic of injected cells

Characterization of the injected cells was performed on cytospin preparations for each cell batch. β -MHC and CD31 immunostaining were used to evaluate the percentage of cardiomyocytes and endothelial cells, respectively, in the cell preparations. After directed differentiation and enrichment by percoll gradient hESC-CM preparations contained 50 to 65% cardiomyocytes (Figure 2A) whereas the control (non-cardiac cell) preparations contained less than 0.1% cardiomyocytes (Figure 2C). Endothelial cells expressing CD31 were rare ($< 0.1\%$) in both cardiac and non-cardiac cell preparations (Figures 2B, D). For further characterization of the hESC-CM preparations, cell aliquots were re-plated on gelatin coated plates. Spontaneous beating foci reappeared in all preparations 2 to 3 days after re-plating. Patch clamp recording performed on those spontaneously beating cells are characteristic of cardiomyocytes (Figure 2F) confirming their cardiac phenotype. β -MHC immunostaining show clear myosin filament organization within the cells (Figure 2E).

Engraftment of human cardiomyocytes into a chronic infarct

We first tested the ability of hESC-CM to survive and form stable grafts when transplanted into a chronic infarct. Six hESC-CM injected rats were sacrificed 1 month after cell injection (i.e. 2 month after I/R; Figure 1). Histomorphometry performed on picrosirius red-stained sections showed that the infarcted area comprised $18.6 \pm 4.7\%$ of the left ventricle. Using β -MHC immunostaining, we detected human cardiomyocytes in all animals, with grafts representing $0.83 \pm 0.22\%$ of the left ventricle (Figures 3A, B, C). Human cardiomyocytes did not show robust sarcomere organization (Figure 3D). Interestingly, many of the vascular structures present in the grafts contained human endothelial cells. The lumens of these microvessels contained erythrocytes (Ter119 positive cells) that they are connected to the host coronary vasculature (Figures 3E, G). The rat endothelium grew into the proximal graft region and was found in close association with the human endothelium. No human-derived endothelial cells were detected outside the cardiomyocyte grafts (Figures 3F). Histologic examination did not reveal teratomas or other inappropriate non-cardiac tissue structures.

At three months after intramyocardial injection, human cardiomyocytes were present in all 13 rats (Figures 4A, B, C) identified by staining and β -MHC and our human pan centromeric in situ hybridization probe. Double immunostaining with β -MHC and BrdU antibodies revealed that human grafts were still proliferative (Figures 4D, E) and had developed immature but readily identifiable sarcomeres (Figure 4F). Cadherin staining was observed between human cardiomyocytes at both 1 and 3 months time points, indicating the presence of adherens junctions between grafted cells. In some cases, cadherin immunostaining was also observed close to the interface between host and graft cardiomyocytes, but we did not observe definitive graft-host junctions (Figure 4F). In one of 13 hESC-CM transplanted rats, we observed cytokeratin-positive epithelial cells forming microscopic cysts of human origin, likely derived from contaminating endoderm (supplementary figure 1). However, close examination did not show teratomas in any heart of the hESC-CM injected rats.

Histologic evaluation of the non-cardiac cell injected cohort was performed at the 3 month time point only. Human cardiomyocytes were detected in 2 of 9 non-cardiac cell injected rats, although all rats in this cohort received injections from the same non-cardiac cell batch. The 2 rats that were positive for human cardiomyocytes were excluded from the functional data analysis. No human cells were detected in the other 7 hearts, indicating death of the grafted cells.

Effect on ventricular function

Baseline echocardiographic evaluation performed in all animals included in the study (hESC-CM $n=13$; PSC $n=15$; non-cardiac $n=7$; and SFM $n=8$) at 3 weeks post-MI (1 week before transplantation) did not show any differences between groups before transplantation, indicating that our inclusion criteria resulted in comparable groups. All groups showed significant chamber dilation and reduced fractional shortening, indicating states of structural remodeling and mechanical dysfunction were present. Further echocardiographic studies were then performed at 1, 2 and 3 months after transplantation surgery. Using a 2 way ANOVA method to analyze the echocardiographic parameters, a global increase in LVEDD and LVESD was observed over time, indicating left ventricular remodeling was still progressing from 3 weeks to 4 months after myocardial infarction ($p < 0.0001$). However, LVEDD, LVESD and FS were similar among groups at each time point of the study (Figures 5A-C, table SI supplementary data), suggesting that left ventricular dimension and function were not affected by cells or PSC injection.

MRI analyses performed in all animals at the 3 months time point also failed to show any difference between the 4 groups (Figures 6A-C, table SII supplementary data). To further

evaluate left ventricular heart function we performed supplementary MRI at the 1 month time point in a subset of animals (hESC-CM, n=10, PSC, n=8; Non cardiac n=6; data not shown). Two way ANOVA analysis with paired data revealed that systolic and diastolic volume of the left ventricle, left ventricular mass and ejection fraction were significantly worsened between the 1 month and the 3 months time point ($p<0.0003$), thus confirming the echocardiographic data indicating progressive left ventricular remodeling. However, in this subset of animals, cardiac MRI did not show any difference among groups in any cardiac parameter at any time point of the study. Taken together, the MRI and echocardiography data strongly indicate that neither hESC-CM, non-cardiac cells or the pro-survival cocktail have a beneficial effect on cardiac function in the rat chronic infarct model.

Discussion

In the present study, the functional effect of hESC-derived cardiomyocytes after transplantation into a chronic model of myocardial infarction in the nude rat was evaluated for the first time. A previous study from our group has shown that these cells are functionally beneficial in an acute model of myocardial infarction [1]. Using a similar protocol, *i.e.* similarly derived cells, culture conditions, injection protocol and the same nude rat recipient with infarcts induced by ischemia-reperfusion, we demonstrated here long term engraftment of cardiomyocytes derived from hESC after injection into scar tissue of chronically infarcted rat hearts. However, longitudinal analyses of heart function using both echocardiography and MRI showed that the engrafted cardiomyocytes are insufficient to restore heart function or to alter adverse remodeling.

One concern in this study was that the chronic myocardial infarct might prove an inhospitable environment for the growth of a new myocardium [11]. Two studies performed in a rat model suggested that cell engraftment is optimal when cells are injected in subacute infarct (1 or 2 week after injury) rather than in an acute or chronic lesion [12,13]. However, Li *et al.* demonstrated that, 48 hrs after intramyocardial injection, bone marrow stromal cell retention was 6- to 8- fold higher in mature myocardial scar than in acute or non-injured animal model [14]. The low survival of these cell types in the chronic infarct has generally been attributed to the presence of scar tissue and its low vascularity [12,13,15]. Interestingly, our studies showed that human cardiomyocytes survived in the rat heart after engraftment into the chronic infarct as well as into 4-days infarcts. In the chronic infarct, graft size was 0.83% of the left ventricle one month after delivery. We previously reported that the same dose of cells gave grafts of 0.48% in the acutely infarcted heart [1]. Thus, engraftment of new stem cell-derived human myocardium in the chronic infarct is feasible. In order to increased cell survival we used a Matrigel-based vehicle for our intramyocardial injection. Although pro-survival cocktail has been proven to significantly increase cell survival, it may impair functional integration of the cells and thereby limit the beneficial effect of the transplanted cells. In our previous study however, we used the same matrigel-base vehicle for the acute myocardial injections. Because we observed a beneficial effect in that study, Matrigel seems unlikely to explain lack of efficacy of hESC-CM injection in our chronic infarct model.

The need for adequate vascularization is increasingly recognized in cardiac cell transplantation [16]. In the acute myocardial injection protocol, we previously found that vascular structures within grafts were mostly host-derived, *i.e.* rat endothelium [1]. In contrast, after injecting apparently comparable preparations in established scars, we observed vascular structures of human origin one month later. These human microvessels contained blood cells, indicating connection with the host circulation. Interestingly, we also observed human microvessels when hESC-CM were injected into uninjured heart [17], suggesting that an acute infarct may be a hostile environment for survival or proliferation of

endothelial cells or their progenitors. In the current study, at the 3 months time point, we did not observe any human-derived endothelial cells (data not shown). The loss of human endothelium may have occurred during maturation of the grafts' wound-like environment (endothelial apoptosis is common as granulation tissue evolves into scar) [18], or those cells may have been killed by immune mechanisms. The humoral and innate arms of the immune system remain intact in the athymic rats. Furthermore, athymic rats develop oligoclonal T cell populations as they age [19,20].

Sarcomere organization was not observed at the 1 month time point, but at 3 months striation were readily observed in some transplanted cardiomyocytes. An unexpected but welcome result was the presence of BrdU-positive human cardiomyocytes, indicating proliferation of the engrafted cells 3 months after transplantation.

In the literature, few studies report benefits to both structural remodeling and contractile function with cell therapy in the chronic phase of infarction. When injected in a rat model of myocardial infarction, bone marrow derived cells or skeletal myoblast injection have been reported to reverse cardiac remodeling, in association with increased neovascularization or attenuated fibrosis respectively [21]. In rat chronic myocardial infarction models, neonatal or fetal cardiomyocytes have been shown to engraft and benefit cardiac performance [22-24]. In addition, mouse ESC-derived cardiomyocytes have been demonstrated to improve heart function when injected several weeks after myocardial injury [25], suggesting the feasibility of ESC-derived cardiomyocytes therapy for heart failure. However, when compared side by side in the same model, a cell therapy product seems to be more effective when provided in the acute or subacute phase of myocardial infarction rather than at the chronic phase of myocardial infarction. For example, Hu *et al.* reported that, in a rat model, optimal benefits on cardiac function were observed when mesenchymal stem cells are injected at 1 week after myocardial infarction rather than 1 hour or 2 weeks after the injury [12]. Similarly, Li *et al.* showed that fetal cardiomyocyte transplantation at 2 weeks post cryoinjury in rats resulted in better left ventricular function improvement compared to transplanting immediately or 4 weeks after myocardial infarction [13]. In our study, the human cardiomyocyte injection protocol that has been shown to alter ventricular remodeling when injected at the subacute phase of myocardial infarction, did not have any beneficial effect when injected in at the chronic phase, confirming the results of the previous comparative studies.

This difference of efficiency between the acute and chronic models raises the question of mechanism of benefit. One possible hypothesis is that in the subacute myocardial infarction models the injected hESC-CM did not contribute directly to the contractile activity but rather released paracrine factors that protected the heart [21,26]. Examples could include molecules that prevent death of host myocytes [27], promote vascularization [28], enhance host cell contractility [7,29] or prevent degradation of the heart's extracellular matrix [30]. Providing such factors at 4 weeks, when extensive remodeling and mechanical dysfunction have ensued, may simply be too late. Our data do not fit as well with the hypothesis that cell therapy works principally through creation of new systolic force-generating units. We would have predicted that new force generation in the infarct would have been beneficial, even in the chronic setting. This could be one limitation of the current xenotransplantation model, where human cells may not keep pace with the rat's rapid heart rate. If so, the human cardiomyocytes might be more effective in species with a slow heart rate.

In summary, this study demonstrates that hESC-CMs injected into the scar tissue of a chronic infarct model still engraft, survive and form striated cardiomyocytes graft similar to observed in acute myocardial infarction studies. However, although hESC-CM transplantation can attenuate the progression of heart failure in an acute model, the same

hESC-CM injection protocol is insufficient to restore heart function or to alter adverse remodeling of a chronic myocardial infarction model.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Study design. See text for details.

Figure 2. Characterization of cell preparations. (A-D) The percentage of cardiomyocytes and endothelial cells were evaluated in cytospin samples of hESC-CM and non-cardiac cell preparations using β -MHC and CD31 immunostaining, respectively. (E) β -MHC pattern in cardiomyocyte *in vitro* before transplantation showed by confocal microscopy. (F) Representative spontaneous action potential recorded *in vitro*, confirming the cardiac phenotype of the employed hESC-CM.

Figure 3.

Histological evaluation of a human myocardial graft at 1 month after transplantation into the rat heart (2 months after ischemia-reperfusion). (A, B, C) Transverse section of the rat heart with representative human intramyocardial graft visualized by hematoxylin eosin staining (A) or β -MHC immunostaining (B, brown chromagen, DAB) or picrosirius red/fast green staining (C). (D-F) Three consecutive tissue sections with higher magnification of a human graft showing the presence of human cardiomyocytes (D, β -MHC immunostaining) and human endothelial cell arranged in a vessel-like structure within the human graft (E, human CD31 immunostaining). Confocal microscopy confirmed the presence of human endothelial cells located only within the human graft (F, red) whereas rat endothelial cells (RECA immunostaining, green) are present in both host and graft tissue. (G) Double immunostaining for human CD31 (green fluorescence) and TER-119 (red fluorescence) showing presence of erythrocytes inside a lumen-like structure formed by human endothelial cells.

Figure 4.

Histological evaluation of a human graft at 3 months after transplantation (4 months after ischemia/ reperfusion injury). (A, B, C) Representative hematoxylin and eosin staining, β-MHC immunostaining and *in situ* hybridization with the human pan centromeric probe (hPCP) of a hESC-CM graft. (D) Double immunostaining for β-MHC (red chromagen) and BrdU (brown chromagen, DAB; arrows) in bright field suggesting proliferation of some human cardiomyocytes 3 months after injection. (E) Higher magnification of β-MHC (green fluorescence) and BrdU (red fluorescence) immunostaining visualized by confocal microscopy. (F) Confocal microscopy confirming sarcomere organization (β-MHC, green fluorescence) in human cardiomyocytes. Cadherin (G, green fluorescence) is present between host (arrow) and human cardiomyocytes. Occasionally, we observed graft cadherin expression in close proximity to host cardiomyocytes (asterisk), but in most cases the grafts were separated from host cardiomyocyte by scar tissue.

Figure 5.

Echocardiographic evaluation of the rat heart function after hESC-CM injection. No differences among groups were detected at any time. LVEDD (Left Ventricular End Diastolic Dimension), LVESD (Left Ventricular End Systolic Dimension), and FS (Fractional Shortening) were measured by a blinded operator at baseline and at 1, 2 and 3 months after cell transplantation.

Figure 6.

Assessment of post-infarct left ventricular function of the rat heart by MRI 3 months after hESC-CM transplantation. No differences in left ventricle dimensions and function were detected between groups. Abbreviations are ESV: end systolic volume, volume of the left ventricle at the end of systole (in mm³); EDV: end diastolic volume, volume of the left ventricle at the end of diastole (in mm³); SV: Left ventricle stroke volume, calculated as difference between EDV and ESV (in mm³); LV mass: left ventricular mass (in mg); EF: left ventricular ejection fraction, calculated from the relative difference in end-diastolic and end-systolic cavity volumes; wall thickening was evaluated at the infarcted segment, at the opposite wall and at mid septum level.