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Arrhythmia Susceptibility in Mice after Therapy with β-Catenin-Transduced Hematopoietic Progenitor Cells after Myocardial Ischemia/Reperfusion

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Key Words
Arrhythmia · Electrophysiology · Hematopoietic progenitor cell line · Gene transfer

Abstract
Background: Hematopoietic progenitor cells (HPCs) can improve cardiac function after myocardial infarction. However, occurrence of arrhythmias is a potential limitation of cell therapy. In this study, we investigated the cardiac electrophysiological properties of ex vivo expanded HPCs, generated by β-catenin gene transfer, after transcoronary delivery in a murine model of ischemia/reperfusion (I/R) injury. ***Methods and Results:*** To assess arrhythmia inducibility of ex vivo expanded HPCs, mice were subjected to I/R and assigned to sham operation (n = 8), I/R (n = 21) and HPC (n = 15) treatment. Six weeks later, mice were subjected to long-term electrocardiogram recording and in vivo transvenous electrophysiological study. After I/R, mice showed a significant prolongation of conduction and repolarization compared with sham-operated mice. There was a marked increase in ventricular ectopic activity in infarcted mice as compared with sham-operated mice. Cardiac electrophysiological parameters and ventricular ectopic activity were not altered in mice treated with HPCs in comparison with control I/R mice. ***Conclusion:*** Transcoronary delivery of genetically ex vivo expanded HPCs did not alter the electrophysiological properties in mice after I/R. Therefore, ex vivo β-catenin-mediated HPC expansion may represent an attractive therapeutic option for cell transplantation treatment of myocardial infarction without electrophysiological side effects.

Introduction

Myocardial ischemia (MI) is a leading cause of heart failure in western countries [1]. Cell transplantation is a promising new therapy, and several studies have shown that transplanted cells can form viable grafts within the host myocardium [2, 3]. Recent studies have revealed that various types of cells are useful in cell transplantation after MI, such as skeletal myoblasts [4–6], smooth muscle cells [7], bone marrow mononuclear cells [8, 9] and hematopoietic progenitor cells (HPCs) [10–12]. Each cell type may have its own profile of advantages, limitations and practicability issues. However, there are several lines of

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evidence suggesting that cell replacement therapy may be accompanied by an increased arrhythmic risk.

Early clinical and experimental studies of myoblast therapy have shown a concerning incidence of ventricular arrhythmias [5, 13, 14]. Consistent with this clinical observation, basic studies have shown that myoblasts do not electrically couple with native myocardium in vivo [15] and, when injected into the myocardium, tend to cluster near injection sites. In contrast, recent studies using bone marrow-derived cell therapy have reported that a significant incidence of arrhythmias has not been observed [8, 10]. Contrarily, in vitro studies on mesenchymal stem cells [16] and embryonic stem cell-derived cardiomyocytes [17] have shown them to be potentially arrhythmogenic. Albeit, a recent report on Langendorff-perfused rat hearts showed no difference in arrhythmia vulnerability after mesenchymal stem cell treatment after MI in comparison with control MI [18].

Furthermore, the relatively low number of progenitor cells and the difficulties of ex vivo expansion required for therapeutic application represents another major practical limitation [19–21]. We recently reported that genetic modification of HPCs with β-catenin (β-catenin-HPCs) provided an unlimited ex vivo expansion of multipotent progenitor cells [22]. Transplantation of β-catenin-HPCs limited infarct size, attenuated left ventricular (LV) remodeling and ameliorated LV function in a cell concentration-dependent manner [23].

In the present study, we specifically investigated the effects of genetically ex vivo expanded HPCs using β-catenin gene transfer on cardiac electrophysiological properties.

Methods

β-Catenin Gene Transfer into HPCs

The cell line was generated and characterized as described previously [22]. Briefly, human β-catenin cDNA was cloned into the retroviral vector SFβ-91-IRES-eGFP, and recombinant VSV-G-pseudotyped retroviruses were generated in the packaging cell line Phoenix-gp. Lineage– (lin–) bone marrow cells were isolated from male C57BL/6 mice using a lineage depletion kit (Miltenyi Biotech, Bergisch Gladbach, Germany). Retroviral gene transfer into lin– bone marrow cells was performed in the presence of a stem cell cytokine cocktail consisting of 10 ng/ml recombinant murine interleukin-3 (rmIL-3), 50 ng/ml recombinant human IL-11 (rhIL-11), 50 ng/ml recombinant human fms-like tyrosine kinase 3 (rhFlt3) and 50 ng/ml recombinant murine stem cell factor (rmSCF; all from Cell Systems, St. Katharinen, Germany) over 48 h and transduced with a multiplicity of infection of 10 with 8 μg/ml polybrene (Sigma, Deisenhofen, Germany). Cells were cultured for 24 months in IMDM containing 10% FCS, 2 mM L-glutamine, 1% penicillin-streptomycin, 1 mM non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol, 10 ng/ml rmIL-3 and 50 ng/ml rmSCF (all from Cell Systems).

Flow Cytometry Analysis

Staining with fluorescent conjugated c-kit-PE and Sca-1-FITC antibodies and corresponding isotype controls (BD Pharmingen, San Diego, Calif., USA) were performed in ice-cold phosphate-buffered saline (pH 7.4) containing 2% FCS for 30 min at 4°C. Cells were analyzed using FACSCalibur and CellQuest software.

I/R, Transcoronary Infusion and Infarct Analysis

All animal studies were in compliance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health and were approved by the state of Lower Saxony.

Male C57BL/6 mice (aged 10–12 weeks) were subjected to I/R and transcoronary infusion of cells as described previously [24]. In brief, the left anterior descending artery was transiently ligated with an 8-0 Prolene slipknot for 120 min followed by reperfusion. Twenty-four hours after I/R, the left carotid artery was cannulized, a transarterial catheter was placed in the aortic root and cells or saline were infused (200 μl volume). Forty-eight hours after I/R, animals were sacrificed, the area at risk and early infarct size were assessed by Evan's blue and 2,3,5-triphenyltetrazolium chloride staining. In a separate group of animals, MI size was determined after 6 weeks in hematoxylin- and eosin-stained sections and quantified by polarized light microscopy (Zeiss Axiovert 100, Quantimet 500MC digital image analyzer).

Ambulatory Electrocardiogram Telemetry

Before electrophysiological study, the mice underwent ambulatory electrocardiogram (ECG) recordings using implantable PhysioTel TA10EA-F20 radiotransmitters (Data Science International, St. Paul, Minn., USA), as we have described previously [25–27]. Radiotransmitters were implanted 6 weeks after sham operation (n = 8), I/R (n = 21) and cell therapy with β-catenin-HPCs after I/R (n = 15). All baseline surface ECG parameters were measured manually with online calipers by 2 investigators independently. Both investigators were blinded to the therapy regimen of the mice studied. Rate-corrected QTc and JTc intervals were calculated as described previously [28]. Records consisting of >10% artifacts were excluded from further analysis. The mean heart rate was computed, and the occurrence of ventricular premature beats (VPBs), supraventricular and ventricular tachycardia and bradycardia were analyzed.

Electrophysiology Study Protocol

Invasive electrophysiological studies were performed after ambulatory ECGs were collected 6 weeks after sham I/R, I/R and I/R with additional cell therapy, respectively. The methodology of mouse invasive electrophysiological study has been previously described in detail by us [25, 26]. Surface ECG interval measurements have been well defined in mice. QT and JT intervals were corrected for heart rate according to the formula proposed by Mitchell et al. [28]. Measurements of surface ECG parameters were performed by consensus between 2 investigators. A 2-french octapolar catheter with an interelectrode interval of 0.5 mm (CIBer mouse EP; NuMed, Nicholville, N.Y., USA) was inserted

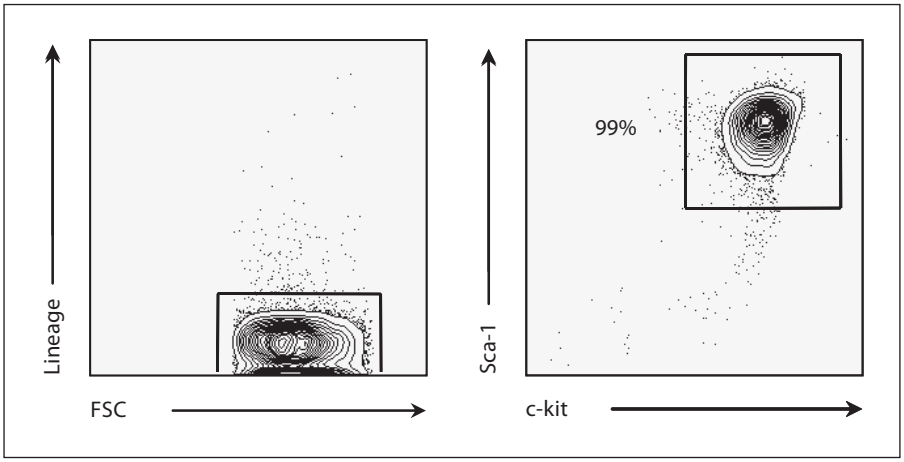

Fig. 1. In vitro characterization of β-catenin-transduced HPCs. Hematopoietic progenitor surface marker profile of β-catenin-HPCs. Fluorescence intensity plots are shown in log scale. FSC = Forward scatter.

via a jugular vein cutdown approach into the apex of the right ventricle of the murine heart. Right ventricular stimulation was performed with the distal electrode and right atrial stimulation with the proximal electrode of the catheter, assuring a constant stimulation site. We used a standardized stimulation protocol for each electrophysiological study in all studied animals. Investigators were blinded to the therapy regime of the mouse studied. Pacing and programmed stimulation were performed in anesthetized mice, as previously reported [25–27, 29].

Delivery of burst extrastimuli was performed in decreasing coupling intervals of 10 ms, until atrial conduction failed to result in ventricular depolarization. Atrial and ventricular refractoriness and atrioventricular (AV) effective and functional refractory periods were obtained using a standard programmed atrial stimulation protocol, as has been well described in humans and mice elsewhere [26, 29]. After assessing standard electrophysiological parameters, double and triple atrial and ventricular testing of S2–S4 followed by burst atrial and ventricular pacing at a cycle length of 30 ms for a maximum of 1 min was applied to assess atrial and ventricular arrhythmia inducibility. The protocol was terminated after arrhythmia was reproducibly inducible. Inducibility of >3 repetitive supraventricular or ventricular beats were defined as nonsustained, >30 s as sustained atrial or ventricular tachycardia. Diagnosis of atrial and ventricular tachycardia in mice is well established and includes analysis of sudden rate change, identifiable P wave, QRS morphology and duration in comparison with sinus rhythm and endocardial atrial and ventricular activation. Differentiation between atrial and ventricular tachycardia was done by consensus between 2 investigators. Mice were excluded from analysis if the catheter could not be adequately placed in the right ventricle or if mice did not recover from anesthesia after the study, indicating right ventricular perforation.

Statistical Analysis

All continuous variables, such as cardiac dimensions, ECG intervals and cardiac conduction properties were compared with sham, with data presented as the mean ± standard deviation. Statistical analysis was performed with ANOVA followed by Bonferroni post-hoc analysis. The occurrence of VPBs was compared with Fisher's exact test for the comparison of categorical variables. Comparison of the incidence of inducible or spontaneous arrhythmia was performed with Fisher's exact test. A p value <0.05 was considered statistically significant.

Results

In vitro Characterization of the HPC Line

β-Catenin-transduced lin– cells survived and proliferated in a cytokine cocktail of IL-3 and SCF for more than 24 months, whereas the viability of eGFP-transduced control cells dramatically decreased after 22 days of culture [22]. β-Catenin-transduced cells stably expressed (>99%) hematopoietic stem cell markers Sca-1 and c-kit (fig. 1), but failed to express lineage markers of myeloid (CD11b, GR-1, CD11c), erythroid (TER119) and lymphoid (B220, CD3e, NK1.1, CD11c) lineages. The constitutive β-catenin expression was gradually downregulated in the HPC line during 40 weeks of culture through an unknown silencing mechanism, whereas β-catenin cDNA was stably integrated in β-catenin-HPCs at 1, 8, 16, 24 and 40 weeks after transduction [22]. In the present study, we used the HPC line after 18 months of generation and culture.

Transcoronary β-Catenin-HPC Injection Reduces Infarct Size

The mortality rate during the whole study period did not differ between the groups of animals treated with HPCs (2/17), sham (1/9) and I/R (3/24).

Six weeks after I/R, the hearts of all mice had anterolateral wall infarction. Injection of 1×10^7 β-catenin-HPCs (n = 7) reduced the infarct size significantly com-

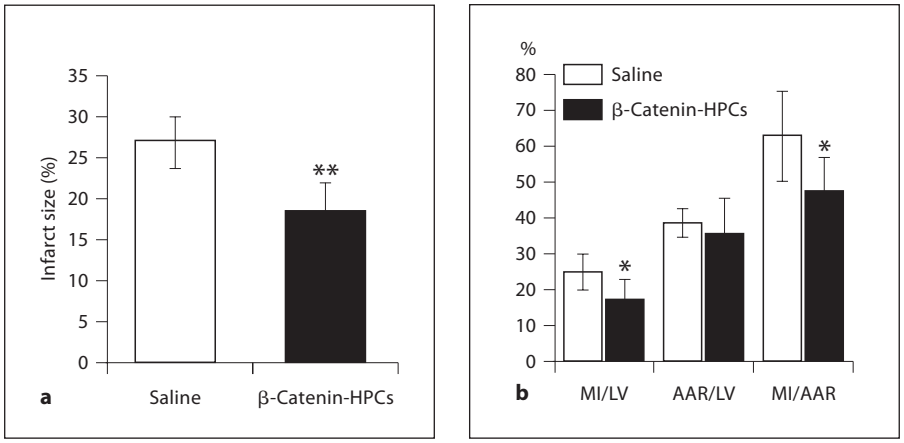

Fig. 2. Transcoronary HPC injection reduces infarct size. **a** Quantification of infarct size by morphometric analysis in saline- (n = 13) and β-catenin-HPC-treated (n = 7) mice. The infarct size was greater after saline injection and was reduced after injection of β-catenin-HPCs. ** $p < 0.01$ versus saline. **b** The area at risk (AAR) and infarcted area (MI) were expressed as percentage of the LV cross-sectional area (LV); MI was also calculated as percentage of AAR. * $p < 0.05$ versus saline.

Table 1. Surface ECG conduction intervals in mice during ambulatory ECG recording

	n	RR ms	P ms	PR ms	QRS ms	QT ms	QTc ms	JT ms	JTc ms
Sham	8	96 ± 4	13 ± 2	38 ± 2	9 ± 1	53 ± 6	50 ± 5	44 ± 6	41 ± 5
I/R	21	100 ± 5	11 ± 4	35 ± 3	15 ± 4**	63 ± 8**	59 ± 5**	48 ± 7	46 ± 5
β-Catenin-HPCs	15	98 ± 5	12 ± 3	36 ± 3	16 ± 3**	63 ± 7**	59 ± 4**	47 ± 8	44 ± 5

RR = RR interval; P = duration of P wave; PR = duration of PR interval; QRS = duration of QRS interval; QT = duration of QT interval; QTc = rate-corrected duration of QT interval; JT = duration of JT interval; JTc = rate-corrected duration of JT interval. ** $p < 0.01$, compared with sham.

pared with saline-injected mice (n = 13; $p < 0.01$; fig. 2a). To determine the effect of β-catenin-HPCs on early infarct size, the hearts were isolated 48 h after I/R and 24 h after transcoronary cell delivery, respectively. The size of the area at risk during left coronary occlusion was comparable in the β-catenin-HPC-treated (n = 6) and saline-injected (n = 6) hearts. However, infarct sizes were significantly smaller in β-catenin-HPC-treated hearts ($p < 0.05$ vs. saline; fig. 2b).

Ambulatory ECG Telemetry

The total number of ambulatory ECGs performed and the results of the electrocardiographic data are summarized in table 1. A significant prolongation of QRS, QT and QTc comparing sham mice with I/R mice was observed. Cell therapy with β-catenin-HPCs after I/R did not significantly alter any ECG parameter as compared with I/R (table 1).

During a of 12-hour Holter recording, there was no significant difference in heart rate between the studied groups. Significantly more (≥100 VPBs/12 h; $p < 0.05$) spontaneous VPBs occurred in mice after I/R as compared with sham controls (fig. 3). After cell transplantation in I/R, there was no significant difference in the occurrence of VPBs as compared with I/R.

Electrophysiological Study Protocol

The total number of animals with completed electrophysiological study and the results of surface ECG parameters of the electrophysiological data are summarized in table 2.

Cardiac Conduction Properties and Electrophysiological Data

There was no significant difference in the groups with regard to sinus node function and AV conduction (AV interval, AV Wenckebach cycle length, AV 2:1, AV effective refractory periods, AV functional refractory periods). The ventricular refractory period was increased significantly in I/R and β-catenin-HPC mice compared with sham controls.

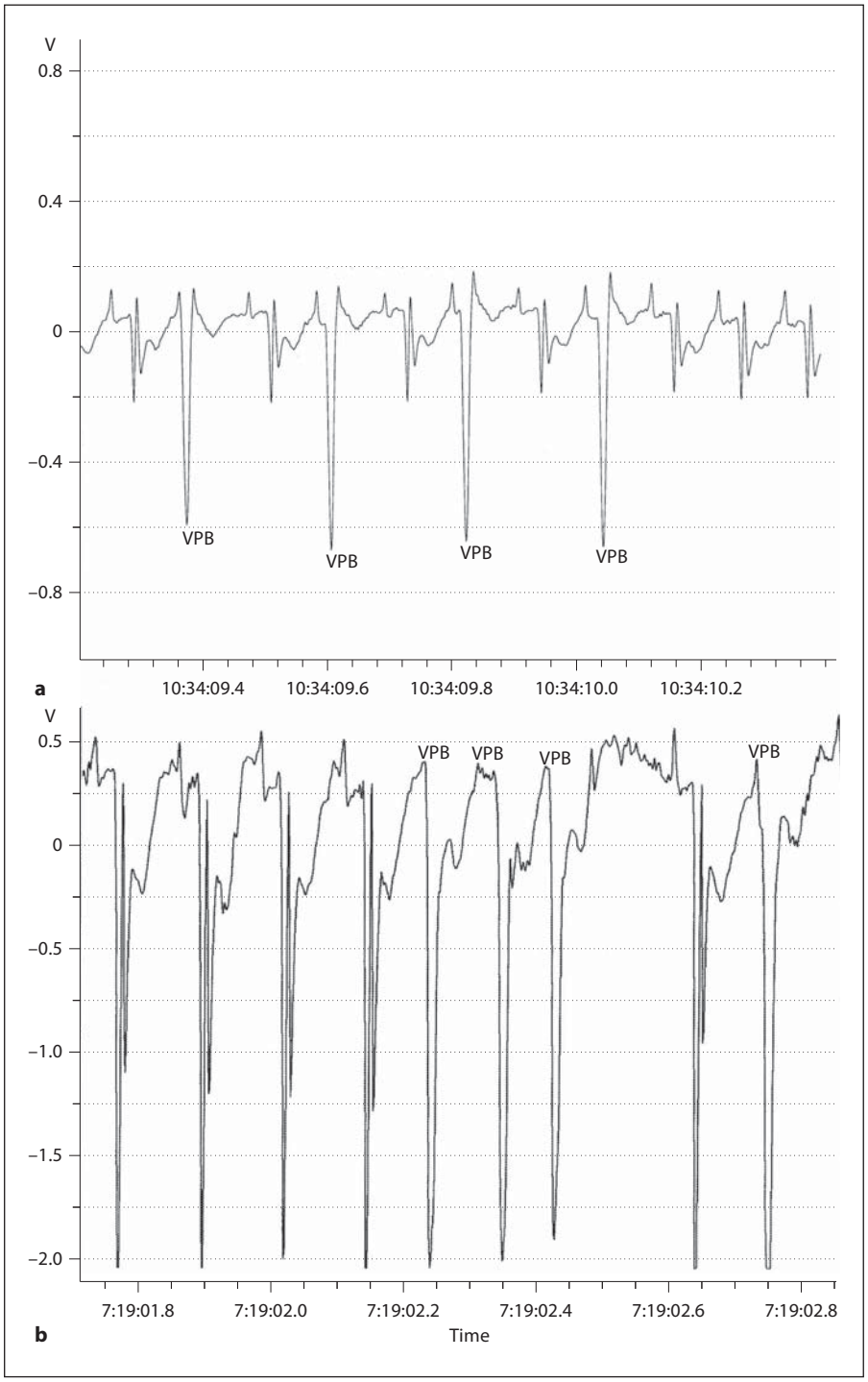

Fig. 3. Long-term ECG recording in mice. Representative recording of VPBs during long-term ECG recording in an I/R (**a**) and β-catenin-HPC (**b**) mouse.

Programmed Stimulation and Arrhythmia Inducibility

With standard programmed electrical stimulation protocols and burst atrial and ventricular pacing, provocation of ectopic or reentrant rhythms was attempted. No animal experienced spontaneous ventricular arrhythmias during placement of the catheter. Nonsustained ventricular tachycardia was inducible in 1 sham and 1 I/R mouse. Therapy with β-catenin-HPCs did not enhance ventricular vulnerability – no ventricular

Table 2. Electrophysiological data summary

	n	SNRT ms	WB ms	AV 2:1 ms	AVERP ms	VERP (S2) ms	VERP (S3) ms	VERP (S4) ms
Sham	7	177 ± 9	94 ± 4	64 ± 8	51 ± 8	25 ± 3	26 ± 2	31 ± 4
I/R	13	178 ± 35	101 ± 9	65 ± 8	56 ± 10	33 ± 8*	33 ± 8*	40 ± 9*
β-Catenin-HPCs	7	185 ± 15	98 ± 4	62 ± 5	55 ± 4	35 ± 7*	36 ± 9*	43 ± 9*

SNRT = Sinus node recovery time; WB = Wenckebach block; AV 2:1 = atrioventricular 2:1 conduction; AVERP = atrioventricular effective refractory period; VERP = ventricular effective refractory period. * $p < 0.05$, compared with sham.

tachycardia was inducible in these mice (0/7 mice). Atrial tachycardia/atrial fibrillation was inducible in 1 sham, 2 I/R and 1 β-catenin-HPC mouse; there was no statistically significant difference in any of the compared groups.

Discussion

In the present study, we show that I/R in mice alters cardiac electrophysiology by prolonging repolarization and ventricular refractoriness as well as by facilitating ventricular automaticity, as has been shown by us and various other groups previously [26, 27, 30, 31]. The major finding of this study is that transaortal high-dose cell transplantation with genetically modified HPCs does not alter cardiac electrophysiology in vivo or enhance ventricular automaticity in mice after I/R. We previously described genetic modification of HPCs with β-catenin, i.e., β-catenin-HPCs, as a strategy for ex vivo expansion of HPCs providing unlimited access for repair of damaged myocardium [22]. Furthermore, we reported that β-catenin-HPC transplantation caused a reduction in MI size, enhanced angiogenesis and antiapoptotic effects, associated with improvement in cardiac function in a cell dose-dependent manner [23]. Thus, cell therapy with genetically modified HPCs after myocardial infarction in mice seems to be safe with regard to ventricular spontaneity and susceptibility to arrhythmias.

Fatal cardiac arrhythmias are a major limiting factor for cell transplantation therapy in myocardial infarction. It is well recognized that transplantation of skeletal myoblasts after myocardial diseases can promote malignant arrhythmias both in animal models and in human studies [5, 32, 33]. It has been postulated that the absence of apparent gap-junctional coupling of skeletal myoblasts to native cardiomyocytes may enhance non-uniformity of conduction, hence precipitating arrhythmias [15, 34].

By contrast, transplanted stem cells show clear histological evidence of gap-junctional formation and contact with host tissue in animal studies, but in vitro studies on mesenchymal stem cells and embryonic stem cell-derived cardiomyocytes have shown them to be potentially arrhythmogenic because they differentiated heterogeneously, were spontaneously active, had abnormal action potentials and showed a high proclivity towards arrhythmia induction [16, 17]. On the other hand, less arrhythmic risk has been seen in human trials involving bone marrow-derived cells, which supports our results [8, 10, 35]. Nevertheless, further histological and electrophysiological mapping experiments have to be performed in future to address these issues.

In the present study, a transcoronary approach after transient MI was chosen to mimic the everyday clinical situation of rescue angioplasty and cell therapy. After the occluded coronary artery was reperfused, cells were infused via a transcoronary approach to ensure that a high concentration of cells reached the infarcted myocardium. Injection of cells directly into the scared myocardium, as has been performed in several animal and human studies previously [13, 14, 17], may promote the forming of isolated islets, which may per se enhance ventricular automaticity and facilitate arrhythmias.

Furthermore, we provide evidence that the transplantation of large numbers of cells is safe in terms of arrhythmogenicity. Limited cell quantity is a major problem for cell therapy with progenitor cells. Previous studies transplanted a few hundred thousand cells per animal after experimental myocardial infarction [30, 32, 36]. Since we previously demonstrated that myocardial recovery after infarction was dependent on the amount of cells transplanted [23], we generated a novel multipotent hematopoietic cell line with a stable expression of Sca-1+ and

c-kit+ by overexpressing β-catenin in HPCs using a retroviral vector to have access to an unlimited amount of cells [22]. Consecutively, as many as ten million cells were transplanted using a transcoronary approach after I/R per mouse.

The possibility that gene-manipulated cells which enhance angiogenesis and have antiapoptotic effects associated with improvement in cardiac function may promote local heterogeneity of ischemic myocardium, resulting in unstable reentry, slowed conduction or altered repolarization, constitutes a potential complication of this therapy. Few studies have previously been performed to evaluate safety of gene therapy in myocardial infarction. In a study with 25 patients undergoing vascular endothelial growth factor gene transfer for therapeutic angiogenesis, no complications were reported [37]. Shintani et al. [38] recently reported a reduction in infarct size after a combined therapy with CD34+ cells and vascular endothelial growth factor 2 gene therapy in a rat model of MI. No major safety concerns and adverse events were reported. Our data indicate that genetic manipulation of HPCs per se does not enhance ventricular automaticity. There was no difference in the electrophysiological properties of mice treated with β-catenin-HPCs after I/R compared with untreated animals. This finding may be explained by the fact that in both groups, there was a cardiac damage and scar. Thus, a substrate for ventricular automaticity was induced by ischemia in both groups of animals, although the LV function was better after cell transplantation. Cell transplantation does not seem to have modified the substrate for arrhythmias since repolarization and ventricular refractoriness were also altered in the same manner.

Limitations

In vivo electrophysiological testing in mice may not be relevantly extrapolated to human clinical diseases, but provide an opportunity for hypothesis testing and understanding the pathology of clinically relevant cardiac electrophysiology. A specific limitation of this study is that we do not present data of a control group, i.e., we did not perform cell therapy experiments with cells without β-catenin transduction. The reason is that such large numbers of c-kit+/Sca-1+ cells can only be obtained by β-catenin gene transfer and the generation of a cell line. Therapy with a significantly lower number of cells would not fulfill the criteria for an appropriate control group. However, as previously reported, the constitutive β-catenin expression was gradually downregulated in the HPC line during 40 weeks of culture through an unknown silencing mechanism, whereas β-catenin cDNA was stably integrated. These results suggest that ectopic expression of β-catenin immortalizes HPCs, but its expression is not required for their survival, proliferation and maintenance. In the present study, we used the HPC line after 18 months of generation and culture. Therefore, a direct influence of β-catenin on cell retention and cellular action cannot be addressed.

Since an I/R mouse model in contrast to permanent myocardial infarction was implemented, infarct size was relatively small. This may have resulted in fewer spontaneous as well as inducible arrhythmias.

Furthermore, no data are presented on the arrhythmogenicity of the ischemic border zone. Additional optical mapping techniques, which would provide mechanistic information on conduction velocity and dispersion, should be performed in future studies to further address this issue.

A further limitation may be the fact that, as previously reported, transcoronary injected cells did not show long-term, sustained engraftment (>24 h) in the scared myocardium. On the other hand, we showed neovascularization in the ischemic myocardium, reduced apoptosis and formation of tube-like structures in vitro in a cell number-dependent manner [23]. Nevertheless, it can be stated that these beneficial effects are not accompanied by adverse alteration of cardiac electrophysiology or enhanced ventricular spontaneity in mice in vivo.

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