Regenerative medicine in cardiovascular disease: from tissue engineering to tissue regeneration
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PART II

Cell Based Regenerative Therapy for
the Treatment of Acute Myocardial Infarction
CHAPTER 5

MESENCHYMAL STEM CELLS FROM ISCHEMIC HEART DISEASE PATIENTS IMPROVE LEFT VENTRICULAR FUNCTION AFTER ACUTE MYOCARDIAL INFARCTION

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ABSTRACT

Background  Mesenchymal stem cells (MSCs) from healthy donors improve cardiac function in experimental acute myocardial infarction (AMI) models. However, little is known about the therapeutic capacity of human MSCs (hMSCs) from patients with ischemic heart disease (IHD). Therefore, the behavior of hMSCs from IHD patients in an immune-compromised mouse AMI model was studied.

Methods  eGFP labeled hMSCs from IHD patients (hMSC group: $2 \times 10^5$ cells in 20 μl, $n=12$) or vehicle only (Medium group: $n=14$) were injected into infarcted myocardium of NOD/scid mice. Sham-operated mice were used as Control ($n=10$). Cardiac anatomy and function were serially assessed using 9.4-T magnetic resonance imaging (MRI); 2 weeks after cell transplantation immunohistological analysis was performed.

Results  At day 2, delayed-enhancement MRI showed no difference in MI size between the hMSC and Medium groups ($33 \pm 2\%$ versus $36 \pm 2\%$; $p=ns$). A comparable increase in left ventricular (LV) volume, and decrease in ejection fraction (EF) was observed in both MI groups. However, at day 14, EF was higher in the hMSC than in the medium group ($24 \pm 3\%$ versus $16 \pm 2\%$; $p<0.05$). This was accompanied by increased vascularity and reduced thinning of the infarct scar. Engrafted hMSCs ($4.1 \pm 0.3\%$ of injected cells) expressed von Willebrand factor ($16.9 \pm 2.7\%$) but no stringent cardiac or smooth muscle markers.

Conclusions  hMSCs from patients with IHD engraft in infarcted mouse myocardium and preserve LV function 2 weeks after acute myocardial infarction, potentially through enhancement of scar vascularity and reduction of wall thinning.
INTRODUCTION

Cell therapy is a promising treatment modality for patients with ischemic heart disease (IHD). Recent, clinical studies showed a beneficial effect on left ventricular (LV) function after application of unselected bone marrow-derived mononuclear cells (BM-MNC) in the setting of acute myocardial infarction (AMI) [1] or in patients with chronic myocardial ischemia [2].

BM-MNCs are a heterogeneous group of cells containing >99.9% committed or differentiated cells, as well as a small fraction of uncommitted cells including mesenchymal stem cells (MSCs) [3]. After injection into ischemic myocardium, bone marrow-derived MSCs from various animal species can differentiate into multiple cell lineages, including endothelial cells [4] and cardiomyocytes [5,6], thereby improving LV function [4,5,6]. In contrast, less information is available about the potential therapeutic capabilities of MSCs from human origin (hMSCs). In rat AMI models, transplantation of hMSCs from healthy subjects across xenogeneic barriers with or without immune suppression improved LV function [7,8]. Also, in the only clinical post-MI trial described to date, Chen et al. found that intracoronary infusion of autologous BM-derived MSCs resulted in an improved LV function [9]. However, little is known about engraftment, differentiation potential and therapeutic capacity of hMSCs from IHD patients after transplantation in an ischemic myocardial environment.

Previous studies demonstrated that BM-MNC and endothelial progenitor cells (EPC) from IHD patients exhibit reduced migratory and colony-forming activities in vitro and resulting in less neovascularization in an ischemic hind limb mouse model as compared to cells from healthy individuals [10]. Also, risk factors for IHD correlate with reduced numbers and functional activity of circulating EPC [11]. At present, it is unclear if the presence of numerous cardiovascular risk factors in IHD patients affects the therapeutic potential of their hMSCs. As a diminished regenerative capability of hMSCs from IHD patients potentially limits their therapeutic efficacy, knowledge on the behaviour of these cells in ischemic myocardium is of interest.

The availability of the immunodeficient NOD/scid mouse strain allows the investigation of hMSCs in animals without pharmacological immune suppression which might interfere with the injected cells [12]. Furthermore, in recent years magnetic resonance imaging (MRI) techniques have been developed allowing for accurate and reproducible determination of murine cardiac volume and infarct size [13]. The high spatial and temporal resolution combined with the non-invasive nature of MRI offer new insights in the dynamic changes induced by cardiac stem cell therapy by serial assessment of cardiac morphology and function.

In the present study, we investigated whether hMSCs from patients with IHD (i) are able to engraft and survive in the acutely infarcted myocardium of NOD/scid mice, (ii) prevent remodeling and improve LV function of the infarcted hearts, and (iii) differentiate into endothelial cells, smooth muscle cells (SMCs) or cardiomyocytes after myocardial engraftment.
METHODS

Animal Experiments

Experiments were performed in 8 to 10 weeks old male immunodeficient NOD/scid mice (Charles River Laboratories, Maastricht, the Netherlands), which lack the ability to mount an adaptive B- and T-cell mediated immune response. All experiments were approved by the Committee on Animal Welfare of the Leiden University Medical Center (LUMC), the Netherlands. Animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

BM Harvest, hMSC Isolation, Expansion and Labeling

hMSCs were purified from leftover BM samples of 4 adult IHD patients with drug-refractory angina and myocardial ischemia who were enrolled in ongoing clinical stem cell trials [2] as previously described [14]. Briefly, BM was aspirated from the posterior iliac crest after local anaesthesia, after which the mononuclear cell fraction (BM-MNC) was isolated by Ficoll density gradient centrifugation. Twenty-four hours after seeding of the BM-MNCs in culture flasks, non-adherent cells were removed and adherent hMSCs were expanded by serial passage. A FACSsort flow cytometer and CellQuest software (Becton Dickinson, Franklin Lakes, NJ USA) was used to characterize the hMSC surface antigen profile, as previously described [15]. hMSCs abundantly expressed hyaluronate receptor (CD44), major T-cell antigen (Thy-1; CD90), endoglin (CD105), vascular cell adhesion molecule-1 (CD106), and human leukocyte class I (HLA-ABC) antigens. These cells also expressed low levels of transferrin receptor (CD71), P-selectin (CD62P), β3 integrin (CD61), neural cell adhesion molecule (CD56), and membrane cofactor protein of the complement system (CD46) at their surface. Furthermore, hMSCs showed 100% differentiation into adipocytes and osteoblasts after appropriate stimulation confirming their mesenchymal and multipotent nature (data not shown).

To facilitate their identification in vivo, hMSCs of passage 4 to 6 were transduced with 100 infectious units (IU) per cell of a fiber-modified first-generation human adenovirus serotype 5 vector (hAd5/F50.CMV.eGFP) encoding the enhanced green fluorescent protein (eGFP) as previously described [15] in the presence of 5 mM sodium butyrate to enhance transgene expression.

Surgical Protocol

Animals were preanesthetized with 5% isoflurane in a gas mixture of oxygen and nitrogen. After endotracheal intubation and ventilation (rate 200 breaths/min, stroke volume of 200 ml,
Harvard Apparatus), a left anterior thoracotomy was performed and the left anterior descending coronary artery (LAD) was ligated. After 15 min, 20 μl culture medium (M199, Eurobio, Cedex, France) containing 2 × 10^5 hMSCs (MI + hMSC group; n = 12) or no cells (MI + Medium group; n = 14) were injected at 5 sites in the infarcted area and border-zones using a 20-μl syringe with a 33 gauge needle (Hamilton Company, Reno, NV). To determine baselines, 10 animals were prepared in a similar manner but without tightening the suture around the LAD (sham group).

**MR Imaging**

Cardiac anatomy and function were serially assessed 2 and 14 days after AMI using a small animal MRI (Bruker BioSpin). The system consisted of a vertical 9.4-T (400 MHz), 89-mm bore nuclear magnetic resonance spectrometer equipped with a shielded gradient set (1 T/m). A birdcage radiofrequency coil with an inner diameter of 30 mm (Bruker BioSpin) was used to transmit and receive the nuclear magnetic resonance signals. Before imaging, mice were anaesthetised as described above. Mice were then placed supine in a coil with a pneumatic pillow for respiration monitoring and maintained at 1 to 2% isoflurane. Electrocardiogram (ECG) electrodes were attached to the left fore limb and right hind limb. Biotrig software (Bruker BioSpin, Rheinstetten, Germany) was used to acquire ECGs and to measure respiratory rates. First, scout images for long axis orientation of the heart were obtained. Next, images containing a four-chamber view were used to plan the short axis images. Image reconstruction was performed using ParaVision 3.02 software (Bruker BioSpin).

**CONTRAST-ENHANCED IMAGING**

To determine myocardial infarct size at day 2 post-MI, contrast-enhanced MRI imaging was employed. To this end, 150 μL (0.05 mmol/ml) gadolinium (Gd)-DPTA (Dotarem) was injected via the tail vein. A high-resolution ECG- and respiratory-triggered 2D fast-gradient echo (FLASH) sequence was used to acquire a set of 18 contiguous 0.5 mm slices in the short-axis orientation covering the entire heart. Imaging parameters were: echo time (TE) of 1.9 ms, repetition time (TR) of 90.5 ms, (25.60-mm)^2 field of view, matrix size of 256 × 256 and a flip angle of 60°.

**LV FUNCTION**

At day 2 and 14 after MI, LV function was assessed. A FLASH cine sequence was used to acquire a set of contiguous 1 mm slices in short-axis orientation covering the entire long axis of the heart. Imaging parameters were as above except for a TR of 7 ms and a flip angle of 15°.
**IMAGE ANALYSIS**

MRI images were analyzed with the MR Analytical Software System (MASS) for Mice (MEDIS, Leiden the Netherlands). The endocardial and epicardial borders were traced manually by 2 independent investigators who were blinded to the experimental groups. End-diastolic and end-systolic phases and the contrast-enhanced area were identified automatically, after which the percentage of infarcted myocardial volume, LV end-diastolic volume (LVEDV), LV end-systolic volume (LVESV) and LV ejection fraction (LVEF) were computed.

**Histological Examination**

At day 15 after MI, the mice were sacrificed, weighed and their hearts and lungs were removed. After measuring their wet weight, the lungs of each animal were freeze-dried. The difference between the wet and dry weight of the lungs was used as a measure of pulmonary congestion. The hearts were immersion-fixed in 4% paraformaldehyde and embedded in paraffin. Serial sagittal sections of 5 μm were cut across the entire long axis of the heart and subsequently mounted on slides. hMSC engraftment was assessed using antibodies (Abs) against eGFP (A11122, Invitrogen), and vascular endothelial cells were detected by platelet/endothelial cell adhesion molecule 1 (PECAM-1, CD31)-specific Abs (clone MEC13.3, Pharmingen), followed by appropriate secondary biotinylated Abs. For visualisation of the eGFP-specific Abs we employed the ABC staining kit (Vector Laboratories) and the PECAM-1-specific signal was amplified using the CSA system (Dako). 3,3’-diamino-benzidine tetrahydrochloride (Sigma-Aldrich) was used as substrate for horseradish peroxidase. Sections were counterstained with Mayer’s hematoxylin. The number of engrafted hMSCs was determined by counting the DAB positive cells with a 20x magnification in every 10th serial section of the entire long axis of the heart. The number of counted cells was multiplied by 10 to obtain an estimate of the total number of engrafted cells in the heart. The hMSC engraftment rate was subsequently calculated by dividing this number by the number of injected hMSCs (2 × 10⁵ cells) and multiplying the result by 100%.

**ASSESSMENT OF CELL DIFFERENTIATION**

To investigate differentiation of eGFP-labelled hMSCs towards endothelial cells, smooth muscle cells (SMC) or cardiomyocytes, serial sections were incubated with Abs against human specific von Willebrand factor (vWF; 4400-5884, Biogenesis), α-smooth muscle actin (ASMA; clone 1A4, A2547, Sigma-Aldrich), human specific smooth muscle myosin heavy chain (smMHC; clone HSM-V, M7786, Sigma-Aldrich), α-sarcomeric actin (clone 5C5, A2172, Sigma-Aldrich), sarcomeric myosin heavy chain (MHC; clone MF20, Hybridoma Bank, Iowa City, IA) and cardiac troponin I (cTnI; clone 19C7, 4T21, HyTest). Primary Abs were visualized with appropriate secondary biotinylated Abs followed by Qdot 655 streptavidin-conjugated (Q10121MP, Invitrogen) Abs.
eGFP-specific labeling was detected with an antibody against eGFP (A11122, Invitrogen) followed by an Alexa Fluor 488 antiserum. Colocalization of eGFP and differentiation markers was examined using a Nikon eclipse E800 fluorescence microscope (NIKON Europe, Badhoevedorp, The Netherlands) equipped with dedicated Q-dot compatible filter sets.

**In Vitro hMSC Culture**

To compare the protein expression of *in vitro* cultured and *in vivo* injected hMSCs, a small fraction of eGFP-transfected hMSCs from the same cell batch that was injected in the mice was propagated *ex vivo*. At the start and termination of the *in vivo* experiment, the cells maintained *in vitro* were analysed by immunofluorescence staining [15] using the same aforementioned antibodies and the Nikon eclipse E800 fluorescence microscope.

**Measurement of Vascular Density**

The effect of hMSC transplantation on vascular density was determined by quantifying the number of PECAM-1 positive vessels per mm² in both the infarcted borderzone and infarcted scar area. Measurements were performed on 3 equidistant sections between the apex and ligature (at the midpoint between the LAD ligature and the apex, between the midpoint and the LAD ligature and between the midpoint and the apex) from 5 animals per group. Per section the number of PECAM-1 positive vessels in 8 equally distributed areas of 0.1 mm² in the infarcted antero-lateral wall of the left ventricle, and 6 equally distributed areas of 0.1 mm² in both borderzone areas were counted in a blinded fashion at a 20x magnification. The values were then expressed as number of vessels/mm². All measurements were performed by two independent examiners who were blinded to the experimental groups, using the Image-Pro Plus software package (Media Cybernetics, Carlsbad, CA).

**Assessment of Infarcted Wall Thickness**

To measure thickness of the infarcted wall in both infarcted groups, planimetric analysis of the same sections used for assessment of vascular density was performed using a drawing microscope (Olympus BH-2, Olympus America Inc.). Wall thickness was measured at 2 separate border-zone areas, at the midpoint of the infarct region and averaged for all 3 measurements. Measurements were performed perpendicular to the infarcted wall.

**Statistical Analysis**

Numerical values were expressed as means ± SEM. Comparisons between the sham, MI+Medium and MI+hMSC group were made using one-way analysis of variance (ANOVA), followed by unpaired t-tests between groups. A p value < 0.05 was considered significant.
RESULTS

Myocardial Infarct Size as Assessed by MRI

Two days after LAD ligation, all mice in the MI+Medium (n = 14) and MI+hMSC (n = 12) group underwent contrast–enhanced MRI with Gd-DPTA (Fig. 1A) to determine the extent of the MI. Infarct size did not differ between the 2 groups (Fig. 1B, 36 ± 2% vs. 33 ± 2% of the left ventricle (p = ns)) indicating that hMSC transplantation had no acute effect on infarct size.

Figure 1. Infarct size as assessed by delayed enhancement MRI. (A) Gd-DPTA-enhanced MR image 2 days after MI (left panel), after tracing the endo- and epicardial borders (middle panel), and after automatic quantification of infarct size by the MASS for mice software package (right panel). (B) No difference in infarct size was found 2 days after MI between the medium and hMSC group.

Cardiac Function as Assessed by MRI

LV function was characterized by serial cine MR imaging 2 and 14 days after LAD ligation in both MI groups, and time-matched sham-operated controls. Representative examples of systolic and diastolic short-axis views after sham operation, as well as 2 and 14 days after MI are shown in Fig. 2.

Figure 2. Typical transverse short-axis MR images of the NOD/scid mouse heart at end-diastole (ED; A-C) and end-systole (ES; D-F) in sham-operated mice (A and D) and in mice 2 (B and E) and 14 (C and F) days after MI.
Two days after MI, in both the MI+Medium and MI+hMSC group a comparable increase in LVEDV (62 ± 2 μL and 58 ± 2 μL; p = ns) and LVESV (43 ± 2 μL and 40 ± 3 μL; p = ns) was measured, as well as considerable decrease in LVEF (31 ± 2 % and 33 ± 3 %; p = ns). All values were significantly different from the Sham group (LVEDV 48 ± 2 μL, LVESV 24 ± 1 μL and LVEF 51 ± 1 %; p < 0.05) (Fig. 3A-C). At 14 days post MI, LVEDV (130 ± 8 μL versus 115 ± 12 μL; p = ns, Fig. 3A) and LVESV (90 ± 12 μL versus 110 ± 8 μL; p = ns, Fig. 3B) increased further in both the MI+Medium and MI+hMSC group although a non-significant trend towards an attenuated LVESV increase was observed in the MI+hMSC group. Of interest, at day 14, a significant difference was found between the LVEF in the MI+hMSC group (24 ± 3 %) and the LVEF in the MI+Medium group (16 ± 2 %, p < 0.05, Fig 3C).

**Figure 3.** Anatomical and functional analysis of hearts from sham-operated animals (Sham), animals receiving medium only (MI + Medium) and animals receiving hMSCs (MI + hMSC) 2 days and 14 days after MI, as assessed by high-resolution 9.4-T MRI. Upper panel: Left ventricular end diastolic volume, Middle panel: Left ventricular end systolic volume, Lower panel: Left ventricular ejection fraction Data are expressed as mean ± SEM. * = p < 0.05 versus time-matched medium-treated mice, † = p < 0.05 versus time-matched sham-operated animals.

**Body and Lung Weight**

Body weight prior to the operation was similar in all 3 groups (sham group: 26.5 ± 0.5 g, MI+Medium group: 26.8 ± 0.5 g and MI+hMSC group: 25.8 ± 0.6 g). Two weeks after MI, body weight in the MI+Medium group decreased significantly with 3.4 ± 1.0 g (-12 ± 4 %) as compared to the sham group, which gained body weight with 0.3 ± 0.4 g (1 ± 1 %; p = 0.007) (Fig 4A). In contrast, the body weight loss of 0.8 ± 1.0 g (-3 ± 4 %; p = ns) in the MI+hMSC group was comparable to the sham group. The loss in body weight in the MI+Medium group was accompanied by an increase in lung fluid (p < 0.05), not observed in the MI+hMSC group (Fig. 4B).
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Figure 4. Change in body weight (A) and amount of pulmonary fluid (B) at 2 weeks after induction of myocardial infarction. There is a significant decrease in body weight 2 weeks after MI in the MI+Medium group but not in the MI+hMSC group (A). The amount of lung fluid was increased in the medium-treated group but not in the mice that received hMSCs (B).* = p < 0.05 versus sham-operated animals.

hMSC Engraftment and Differentiation

Two weeks after transplantation, an engraftment rate of 4.1 ± 0.3 % (n = 5) of eGFP-labelled hMSCs was identified in hearts of the hMSC treated animals (Fig. 5). They were detected predominantly in the infarcted antero-lateral wall and border zone of the infarcted area. No hMSCs were present in the non-infarcted posterior and septal walls. Serial sections were assessed to identify eGFP positive cells co-expressing differentiation markers. The infarcted myocardium contained hMSCs positive for the human specific endothelial cell-specific protein vWF (16.9 ± 2.7 %) and the SMC marker ASMA (78.3 ± 4.0 %) (Fig. 7). The engrafted hMSCs did not stain positive for the highly specific human SMC marker smMHC or the cardiomyocyte-specific proteins α-sarcomeric actin, cTnI and MHC (Fig. 7). No eGFP-labelled cells were found incorporated into blood vessels.

Figure 5. Immunohistochemical staining of eGFP-labeled hMSCs from IHD patients in the antero-lateral wall 2 weeks after intramyocardial injection shows substantial engraftment of injected cells.
In Vitro Differentiation of hMSCs

Before injection, hMSCs already stained positive for ASMA, but not for vWF, smMHC, cTnI or sMHC (data not shown). Two weeks after in vitro culture the cells were still positive for ASMA (Fig. 8), but not for any other marker described above. This finding indicates that the ischemic in vivo environment may be responsible for the expression of vWF genes in the injected transplanted hMSCs.

Figure 6. Representative photographs of PECAM-1 staining of the infarct scar in animals treated 2 weeks earlier with medium only (panel A) or with hMSCs from IHD patients (panel B). Infarct scar and borderzone vascularity was significantly increased in the hMSC group as compared to the medium group (C), and was associated with an reduction in infarct scar wall thinning at 2 weeks post MI (D). * = p < 0.05 versus time-matched medium-treated mice.
Figure 7. Assessment of differentiation of intramyocardially injected hMSC from patients with IHD towards endothelial cells, SMCs or cardiomyocytes using immunofluorescence microscopy. The left column shows engrafted hMSCs in green (Alexa 488) and nuclei in blue (Hoechst), the middle column shows staining of the indicated protein expression in red (Qdot 655) and the right column is a merge of both images. Yellow arrows indicate host vascular media stained by ASMA.
Vascular Density and Wall Thickness

Vascular density in the infarcted scar area and border-zone areas was compared between the 2 MI groups, 14 days after cell administration. Vessel diameter ranged from 8 μm to 94 μm in diameter in all groups. The total blood vessel density (as determined by the number of PECAM-1 positive vessels per mm²) in the scar area was significantly higher in the MI+hMSC group (610 ± 78 / mm², n = 5) than in the MI+Medium group (347 ± 56 / mm², n = 5; p < 0.05; Fig. 6A, B and C). Furthermore, also in border-zone areas of the infarcted hearts, vessel density was increased in the MI+hMSC group (810 ± 68 / mm²) as compared to the MI+Medium group (565 ± 50 / mm², p < 0.05; Fig. 6C). In addition, measurements of wall thickness showed that hMSC injection significantly reduced the extent of infarct wall thinning (MI+Medium group: 18.0 ± 2.1 × 10⁻² mm, MI+hMSC group: 30.5 ± 2.9 × 10⁻² mm; p < 0.05; Fig. 6A, B and D).

Figure 8. hMSCs from the batches that were used for the transplantations exhibit ASMA (upper panel) but not vWF (lower panel) staining after in vitro culture for the duration of the animal experiment. Prior to transplantation hMSCs already expressed the ASMA gene (data not shown). Nuclei are stained blue; ASMA and vWF are stained red.
DISCUSSION

Key findings of the present study are that in an immune-compromised mouse model of acute MI, intra-myocardial injection of hMSCs from patients with IHD resulted in (i) a significant preservation of LVEF in comparison to medium-treated animals, (ii) no limitation of the early infarct size (iii) an increased vascularity of the infarct scar (iv) a marked reduction in the thinning of the infarcted wall, and (v) differentiation of hMSCs towards endothelial cells but not towards cardiomyocytes or SMCs. The present data therefore demonstrate the feasibility of IHD patient derived hMSCs in cell based therapy for acute myocardial infarction.

Although a beneficial effect of autologous MSC transplantation in different animal models of IHD was demonstrated in several studies [4,5,6,16], little information is available about the therapeutic potential of hMSCs from patients with IHD. This is of particular interest because recent studies demonstrated that as compared to healthy controls, human BM-MNCs from patients with IHD have a reduced neovascularization capacity [10], and that risk factors for coronary artery disease correlate with reduced numbers and functionality of circulating hEPCs [11]. In other words limited functionality of cells from IHD patients may limit the potential use of these cells in the treatment of patients with AMI.

LV FUNCTION AND ANATOMY AFTER HMSC TRANSPLANTATION

In the present study, cardiac function and morphology were assessed with a high-resolution 9.4T MRI scanner. MR imaging is a non-invasive technique that uses intrinsic contrast and, unlike one-dimensional (M-mode) and two-dimensional echocardiography, is capable of obtaining true 3-D anatomical and function information. Combined with the high temporal resolution, which enables accurate assessment of cardiac function, MRI is the imaging modality of choice to study the effects of stem cell therapy [17]. In addition, the established clinical MR technique of infarct size determination by delayed contrast enhancement imaging after Gd-DPTA was recently adapted and validated in the mouse MI model [13]. In the present study, no difference in infarct size between the hMSC and medium groups 2 days after MI was found. This was consistent with the functional data, showing at 2 days after MI no difference in any of the functional parameters between the MI+Medium and MI+hMSC groups. These results are in line with the recent findings that after transplantation of hMSC from healthy volunteers in acutely infarcted rat hearts, hMSC had no effect on LV function and remodeling measured 3 days after transplantation by 2D-echocardiography [7]. However, at 2 weeks after MI, a significant preservation of LVEF in the MI+hMSC group as compared to the MI+Medium group was observed. Furthermore we found a non-significant trend towards less LVESV increase in the MI+hMSC group as compared to the non-treated group.

The deterioration in cardiac function in the MI+Medium group was associated with a significant
increase in lung fluid, indicative of pulmonary congestion. In contrast, after hMSC treatment the amount of lung fluid was similar to sham-operated animals. Furthermore, the medium group showed a significant loss of weight of more than 12%, in contrast to only 3% in the hMSC group. Cardiac cachexia is defined as a weight loss of more than 7.5% of the original body weight, and is a sign of end stage heart failure [18]. Therefore, these findings further substantiate the beneficial effects of hMSC injection from IHD patients on the preservation of LV function after AMI.

**Phenotypical Characterization of Transplanted hMSCs**

VWF-positive donor cells were detected after injection of hMSCs in infarcted mouse hearts whereas *in vitro* cultured hMSCs from the same batch that was used for the *in vivo* experiments were negative for vWF at 2 weeks post AMI. Therefore, it is likely that hMSCs acquired this endothelial cell marker in the ischemic myocardial environment. This finding is consistent with a previous study from Zhang et al., in which healthy human donor cells stained positive for vWF at 60 days but not at 3 days after injection of hMSCs in the acutely infarcted myocardium of immune suppressed rats [7]. In addition, several studies reported the differentiation of animal MSCs into endothelial cells after acute and chronic myocardial ischemia [4,6].

In this study, hMSCs also stained positive for ASMA, which is commonly used as a marker for smooth muscle cells (SMCs). ASMA-positive donor cells were detected after transplantation of MSCs in the ischemic rat [19] and canine [4] heart. It was striking however, that in our study the hMSCs were already positive for ASMA prior to their injection and kept expressing the ASMA gene *in vitro* for at least the duration of the animal experiments. In agreement with our findings, Cai et al. showed by immunohistochemistry and Western blot analysis that lapine and canine MSCs in monolayer cultures had ASMA incorporated into stress fibbers [20]. Furthermore, reverse transcription-polymerase chain reaction data showed that *in vitro* cultured hMSCs already contain ASMA-specific transcripts [15]. ASMA gene expression may thus be considered to be intrinsic to these cells. At 2 weeks after transplantation the hMSCs did not stain positive for smMHC, which is a highly specific marker for SMCs [21]. We hence conclude that transplanted hMSCs did not acquire a true SMC phenotype. It cannot be excluded that the injected hMSCs have acquired characteristics of myofibroblasts, which have also been described to be ASMA positive but negative for smooth muscle myosin [22].

In the present study, none of the engrafted hMSCs were positive for the cardiac proteins cTnI, α-sarcomeric actin or MHC. This is consistent with a previous study in dogs where 4 weeks after MI none of the engrafted canine MSCs expressed the muscle-specific gene encoding desmin or the cardiac marker gene cardiac troponin T [4]. Interestingly, Dai et al. recently demonstrated in a chronic rat myocardial infarction model that allogeneic MSCs did not express muscle-specific marker genes two weeks after injection, but did stain positive for the (striated) muscle markers α-actinin, sMHC, phospholamban and tropomyosin [6] after 6 months.
In contrast, Mangi et al. demonstrated that autologous MSCs expressed MHC, cTnI, α-sarcomeric actin and MLC 3 weeks after acute MI in a rat model [23]. Furthermore, other studies also demonstrated cardiomyogenic differentiation of porcine [5], and human [7] MSCs within 2 weeks after transplantation. The MSCs in these latter studies were obtained from healthy subjects. Whether in our experiments the failure of injected hMSCs to differentiate into cardiomyocytes is due to the fact that these cells were obtained from IHD patients is at present not clear, and warrants further investigation.

**POSSIBLE MECHANISMS OF PRESERVATION OF LV FUNCTION BY HMSCS FROM IHD PATIENTS**

Since no transdifferentiation of hMSC into cardiomyocytes was observed, it is likely that hMSCs exert their cardioprotective effects via other mechanisms than cardiomyocyte regeneration. Interestingly, in a recent study of Nakamura et al. injection of long-term cultured porcine MSCs into the acutely infarcted NOD/scid mouse heart resulted in functional improvement at 2 weeks despite minimal differentiation into cardiomyocytes (≈ 0.2 % of the engrafted cells expressed cTnT) [24]. The authors found an increased capillarity in the peri-infarct area, and hypothesized that a possible trophic mechanism must be the basis of the observed beneficial effects [24].

In mice treated with hMSCs from IHD patients, we observed that vessel density in the infarcted scar and borderzone was significantly higher than in medium-treated animals. Although we found some evidence for the endothelial differentiation of hMSCs, none of these cells were incorporated into vascular structures in the scar area. This indicates that in our study the endothelial covering of the vessels in the scar is derived from host tissue. In contrast, in other studies transplanted MSCs that expressed endothelial markers were found in the vessel lining [4,19]. The reason for this discrepancy, however, is unclear. Of note, since in the present study vascular density was assessed 14 days after MI, it could be confounded by an inflammatory response to the transplanted hMSCs. However, the use of the immune-compromised NOD/scid mouse model in the present study makes this less likely. Furthermore, Zhang et al. demonstrated in a rat model of acute MI that an increase in vascularization could be observed as early as 7 days after transplantation of hMSC from healthy volunteers, in contrast to the transplantation of human fibroblasts [25].

The transplanted MSCs may also have preserved LV function by secreting cytokines acting in a paracrine fashion. Recently, it was shown that rat MSCs, engrafted in ischemic myocardium, secrete angiogenic factors including stromal cell-derived factor-1α (SDF-1α), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor(bFGF), which may explain the increase in capillary density [16]. The MSC-mediated increase in vascular structures may increase blood flow within the infarcted area and border zone and thus contribute to (i) salvage of the ischemic myocardium, (ii) inhibition of cardiac remodeling, and (iii) a gradual recruitment of hibernating
cardiomyocytes, and a subsequent improvement in systolic function. From human studies it is known that, significant changes in LVEF can be observed as early as 10 days after revascularization of hibernating myocardium [26]. The observed attenuation of infarct wall thinning in the hMSC group may result from inhibition of cardiac remodeling due to the improved myocardial perfusion. In turn, this may lead to a decrease in wall stress, and subsequently, decreased O2 consumption [27]. This phenomenon may also account for the improved contractile performance of the hMSC treated hearts.

The increased vascularity observed in this study is in line with the improved myocardial perfusion found in clinical studies with BM-MNC [2].

LIMITATIONS

One of the limitations of the present study is the lack of a control group with hMSCs derived from stringently matched healthy subjects, which limits conclusions about a possible beneficial or detrimental effect of the presence of IHD per se on hMSCs therapeutic potential. It should be noted however that even among healthy volunteers, considerable variation in stem cell characteristics may exist, potentially hampering an accurate delineation of IHD-induced alterations in stem cell function from normal biological variation [28]. Furthermore, the aim of our study was to determine the feasibility of using hMSC from IHD patients. This research does not aim however to perform a detailed comparison of these cells to hMSCs derived from healthy individuals. Nevertheless, future studies should be performed to assess the potential differences in therapeutic potential between hMSCs from IHD patients and healthy individuals. Another limitation of this study is the use of a model of acute MI with permanent ligation of the LAD was used, which does not reflect contemporary medical practice where patients with an MI undergo early reperfusion of the culprit artery. However, this reproducible model has been well established in literature and allows comparison with previously reported data. Furthermore, only the short-term effects (2 weeks) of hMSC transplantation were studied. We used MR imaging to assess anatomy and function, which resulted in a rather high mortality because of the long acquisition times under general anaesthesia. This was especially the case in the animals with severe heart failure, making longer-time follow-up with the current protocol practically impossible. Nevertheless, long term studies are warranted.
CONCLUSIONS

hMSCs from patients with IHD engraft in infarcted mouse myocardium and preserve LV function two weeks after acute myocardial infarction, potentially through enhancement of scar vascular-ity and reduction of wall thinning. hMSCs were found to express endothelial cell markers, but no stringent cardiac or smooth muscle markers.

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