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REGULAR ARTICLES

Reduction of infarct size by intravenous injection of uncultured adipose derived stromal cells in a rat model is dependent on the time point of application

A. van Dijk [a,f,g,*], B.A. Naaijkens [a,f,g], W.J.F.M. Jurgens [e], K. Nalliah [a], S. Sairras [a], R.J. van der Pijl [a], K. Vo [a], A.B.A. Vonk [d,f], A.C. van Rossum [b,f], W.J. Paulus [c,f], F.J. van Milligen [a], H.W.M. Niessen [a,d,f]

[a] Department of Pathology, VU University Medical Centre, Amsterdam, The Netherlands
[b] Department of Cardiology, VU University Medical Centre, Amsterdam, The Netherlands
[c] Department of Physiology, VU University Medical Centre, Amsterdam, The Netherlands
[d] Department of Cardiac Surgery, VU University Medical Centre, Amsterdam, The Netherlands
[e] Department of Plastic Surgery, VU University Medical Centre, Amsterdam, The Netherlands
[f] ICaR-VU, Institute of Cardiovascular Research, VU University Medical Centre, Amsterdam, The Netherlands
[g] Technology foundation STW, Utrecht, The Netherlands

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Abstract Stem cell therapy is a promising tool to improve outcome after acute myocardial infarction (AMI), but needs to be optimized since results from clinical applications remain ambiguous. A potent source of stem cells is the stromal vascular fraction of adipose tissue (SVF), which contains high numbers of adipose derived stem cells (ASC). We hypothesized that: 1) intravenous injection can be used to apply stem cells to the heart. 2) Uncultured SVF cells are easier and safer when cultured ASCs. 3) Transplantation after the acute inflammation period of AMI is favorable over early injection.
For this, AMI was induced in rats by 40 min of coronary occlusion. One or seven days after AMI, rats were intravenously injected with vehicle, 5×10^6 uncultured rat SVF cells or 1×10^6 rat ASCs. Rats were analyzed 35 days after AMI.
Intravenous delivery of both fresh SVF cells and cultured ASCs 7 days after AMI significantly reduced infarct size compared to vehicle. Similar numbers of stem cells were found in the heart, after treatment with fresh SVF cells and cultured ASCs. Importantly, no adverse effects were found after injection of SVF cells. Using cultured ASCs, however, 3 animals had shortness of breath, and one animal died during injection. In contrast to application at 7 days post AMI, injection of SVF cells 1 day post AMI resulted in a small but non-significant infarct reduction ($p=0.35$).
Taken together, intravenous injection of uncultured SVF cells subsequent to the acute inflammation period, is a promising stem cell therapy for AMI.

* Corresponding author at: ICaR-VU, Department of Pathology, VU University Medical Centre, De Boelelaan 1117, 1081 HV Amsterdam, The Netherlands. Fax: +31 20 4442964.
E-mail address: Annemieke.vandijk@vumc.nl (A. van Dijk).

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Introduction

Stem cell therapy forms a promising tool to decrease infarction size and to restore contractile function after myocardial infarction. Stem cells namely cannot only improve neovascularization, but theoretically can also replace lost cardiomyocytes, since they have the capacity to differentiate into cardiomyocytes (Lu et al., 2004; Orlic et al., 2001; Wang and Sjoquist, 2006). However, to effectively restore contractile function of the heart after AMI, sufficient stem cells need to home to and remain in the infarcted area and subsequently have to differentiate into cardiomyocytes. Unfortunately, recent studies suggest that only low numbers of stem cells applied to the heart invade the infarcted area and ultimately differentiate into cardiomyocytes (Lee et al., 2007). Therefore more research is needed to improve stem cell therapy.

A potent source of stem cells to improve outcome after AMI is the stromal vascular fraction of adipose tissue (SVF), which contains adipose derived stem cells (ASC). ASCs have properties similar to bone marrow mesenchymal stem cells as has been shown in vitro and in vivo (Zhu et al., 2008; Zuk et al., 2002), and have the capacity to differentiate towards cardiomyocytes (Gaustad et al., 2004; Rangappa et al., 2003; Zuk et al., 2002). In addition, cultured ASCs have already been shown to improve left ventricular function in a porcine AMI model (Valina et al., 2007). Currently a clinical trial (APOLLO) investigates the safety and feasibility of ASCs transplantation in humans (Meliga et al., 2007). However, although these studies are very promising, further optimization is needed. Compared to bone marrow, adipose tissue provides up to 100 times more stem cells per gram, which facilitates the use of uncultured stem cells, or the whole SVF population, as such for transplantation purposes after AMI (Oedayrajsingh-Varma et al., 2006; Zhu et al., 2008). This is advantageous for clinical practice, since culturing is time consuming and expensive (Oedayrajsingh-Varma et al., 2006), and may affect the functional characteristics of stem cells (Rombouts and Ploemacher, 2003), which theoretically may lead to complications during transplantation (Zhu et al., 2008). In this study we therefore compared injection of fresh SVF cells with cultured ASCs in a rat model of AMI.

In addition, we investigated the optimal time point for stem cell delivery, since the time after reperfusion dictates the cellular and humoral environment after AMI, which in turn is an important determinant of the efficacy of stem cell therapy (Azarnoush et al., 2005; Wang and Sjoquist, 2006). During the acute phase of infarction massive myocardial necrosis and leukocyte infiltration may namely harm survival of implanted cells (Lu et al., 2004). Furthermore, it is known that homing and differentiation of stem cells after AMI depends on adhesion factors formed at the site of injury (Azarnoush, 2005; Malek et al., 2006; van Dijk et al., 2008a). It is therefore important to apply stem cell therapy at a time point after infarction when the environment is most favorable for stem cell survival, adhesion and cardiomyocyte formation. However, to the best of our knowledge, this ideal time frame has not yet been determined. In most animal studies, investigators namely apply stem cells during the same operational procedure as the infarct induction. Whereas recent studies have shown that later time points may be more favorable for the survival of the stem cells (Bermejo et al., 2006; Lu et al., 2004; Wang and Sjoquist, 2006). We therefore hypothesized that transplantation after the acute inflammation period (7 days after AMI) would be favorable over early injection (within 1 day after AMI). Taken together, in this study we wanted to compare the effects of intravenous injection of fresh SVF cells and cultured ASCs at different time points on infarct size and outcome in AMI.

Materials and methods

Isolation of the stromal vascular fraction (SVF) of adipose tissue

Adipose tissue from the subcutis and the inguinal fat pad of male Wistar rats (Harlan Laboratories, Horst, The Netherlands; 300–400 g) were stored in sterile phosphate-buffered saline (PBS) and processed within 2 h after surgery. Resected material was minced, and washed with PBS. The extracellular matrix was enzymatically digested with 0.0125% Liberase Blendzyme III in PBS (Roche Molecular Biochemicals, Indianapolis, USA) under intermittent shaking for 30 min at 37 °C. To obtain a single cell suspension, cells were passed through a 200-μm mesh (Braun/Beldico, Marche-en-Famenne, Belgium). Cells were washed with DMEM (BioWhitaker, Cambrex, Verviers, Belgium) containing 10% FBS (Gibco, Invitrogen, California, USA). Hereafter, cells were pooled, and either directly frozen or cultured to second passage, and then frozen under "controlled rate" conditions and stored in liquid nitrogen until needed in experiments.

Cell culture and labeling

SVF cells were seeded at 1×10^5 cells/cm^2 and cultured for two passages in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco, normal culture medium), in a humidified atmosphere of 5% CO_2 at 37 °C.

For cell tracking in vivo, cells were labeled with CM-DiI labeling (Molecular Probes, Eugene, OR USA) according to manufacturer's protocol. In brief, 1×10^6 cells were incubated at 37 °C for 20 min with 5 μl of cell labeling solution in serum free DMEM. Then cells were washed, vortexed for single cell suspension, and injected intravenously in 500 μl of PBS. Single cell suspension was checked during cell counting in a counting chamber and using FACS analysis.

Characterization of rat SVF cells

To study the characteristics of the rat SVF cells and to compare this with human SVF cells, cells were analyzed using colony forming unit assay, as described previously (Oedayrajsingh-Varma et al., 2006). To assess the frequency of colony forming units in the SVF of adipose tissue, SVF cells were seeded in normal culture medium in 6 wells plates at a density of 10^4 and 10^3 cells per well (in triplo). After 14 days cells were washed in PBS, fixed with 4% formaldehyde for 10 min, and subsequently stained in a 0.2% toluidine blue solution in borax buffer for about 1 min. After washing with water, colonies were counted.

To determine the effect of culture on cell size, SVF cells and p1-4 ASCs were compared using a SCEPTER handheld automated cell counter (Millipore, Billerica, MA, USA). To assess population doubling time during culture, passages 1–4 ASCs were seeded at 2.5×10^3 cells/cm^2 in normal culture medium. After incubation for 24 h, unattached cells were removed by washing with PBS. When ASCs reached 70–80% confluence, the number of cells present was determined and plotted against the corresponding time point.

Using fluorescence-activated cell sorting (FACS; FACSCalibur, Becton Dickinson, BD, USA), rat SVF cells and passages 1–4 ASC were phenotypically characterized. Cells were washed in FACS buffer (PBS containing 1% BSA and 0.05% sodium azide) and 10^5 cells were incubated 30 min at 4 °C with conjugated monoclonal antibody of the immunoglobulin G1 (IgG1) isotype in FACS buffer. Antibodies used were FITC labeled monoclonal antibodies against CD166 (Activated Leukocyte Cell Adhesion Molecule, 1:10, clone 3A6, RDI, Flanders, NJ, USA), rat CD90 (Thy 1, 1:25, Cedarlane, Ontario, Canada). Phycoerythrin (PE) conjugated mAbs were used against rat CD31 (MCA1334PE; 1:10, AbD Serotec, Oxford, UK), rat CD45 (leukocyte common antigen, 1:20, Clone OX-1; BD), and CD105 (1:10, Caltag, Invitrogen, Carisbad, CA, USA). Non-conjugated mAbs were used against rat CD34 (Ico115, sc7324; 1:10, SantaCruz, CA, USA), CD54 (eBioscience, San Diego, CA, USA), rat CD73 (Clone 5 F/B9; 1:15, BD), with a secondary incubation (30 min) with a mAb FITC labeled goat-α-mouse IgG1 (1:20, BD). Cells were washed twice with FACS buffer. Nonspecific fluorescence was determined by incubating cells with conjugated mAb anti-human IgG1 (DakoCytomation). Aldehyde dehydrogenase activity, a commonly used characteristic for stem cells (Moreb, 2008), was measured using an aldefluor kit (Stemco Biomedical, Durham, NC, USA) according to the manufacturer's protocol. In brief, samples containing 10^5 cells were labeled with 2.5 μl Aldefluor with or without 5 μl Aldefluor Blocker for 45 min are 37 °C.

Acute myocardial infarction induction

This study was approved by the VU animal ethics and welfare committee. AMI was induced in female Wistar rats (Harlan Laboratories, Horst, The Netherlands, 240–280 g) as described previously (Oedayrajsingh-Varma et al., 2006). In short, a left thoracothomy was performed between the fourth and fifth rib, and a suture was made around the left anterior descending coronary artery. Ischemia was maintained for 40 min, followed by reperfusion.

Before transplantation, the rats were randomly divided into treatment groups and anesthetized using 3% isoflurane. Subsequently, echocardiography was performed. Thereafter, rats received an intravenous bolus of 5×10^6 SVF cells (n=12), 1×10^6 ASCs (n=8) or vehicle (n=11), as harvested earlier from syngeneic rats. Please note that a higher number of SVF cells than ASCs was used, since the SVF is a heterogenous cell mixture, containing 1–10% of ASCs (Oedayrajsingh-Varma et al., 2006). During intravenous injection 3 rats within the ASC group showed shortness of breath during transplantation, one of which died from pulmonary embolism. This rat was excluded from further analysis. Thirty-five days after AMI rats were sacrificed, subsequent to echocardiography of the heart. The latter was performed using 2D-echocardiography, with a 13 MHz linear-array transducer (ProSound SSD-4000 PureHD, Aloka, Tokyo, Japan). Fractional shortening was then calculated using parasternal short axis left ventricular systolic and diastolic diameters. Stroke volume was calculated using doppler velocity time index in the apical view of the aorta.

Histological staining

Infarct size was calculated with a phosphotungstic acid hematoxylin (PTAH) staining and Q-prodit software, as described previously (van Dijk et al., 2009). In short, three equal slices from below the suture were fixed in 4% formaldehyde and embedded in paraffin. Then a PTAH-histological staining was performed on 4 μm sections. This procedure stains the viable area blue, and the infarcted area pink (Fig. 2), allowing infarct size measurements. Infarct size, total left-ventricle myocardial area and wall thickness were quantified using Q-PRODIT analysis (Leica microsystems, Cambridge, UK). In addition, all other organs were analyzed using HE and elastic van Gieson (EVG) staining.

Immunohistochemistry and immunofluorescence

Since paracrine effects of stem cell therapy can decrease local inflammatory response and thereby improve cardiac outcome (Guo, 2007), we also examined inflammatory cells. We have previously shown that neutrophilic granulocytes could hardly be detected 28 days after infarction, in contrast to the number of macrophages that was high within, but not outside, the infarcted area (van Dijk et al., 2009). We therefore only analyzed macrophages in the infarcted area. For this, immunohistochemistry was performed on paraffin slides using a mouse-anti-rat-CD68 antibody (1:100 dilution, 60 min, MCA341R Serotec, Kidlington, UK), after antigen retrieval with 0.1% pepsin (in 0.02 M HCl, 30′, 37 °C). On freeze sections immunohistochemistal staining was performed using an anti-rat-CD31 antibody (1:400 AbD Serotec), and an anti-smooth muscle actinin-antibody (SMA, 1:400, DakoCytomation). As secondary antibody EnVision-HRP (1:200, DakoCytomation) was used. Staining was visualized using EnVision-diaminobenzidin (DakoCytomation). Control sections were incubated with PBS instead of the primary antibody, and yielded no staining at all (not shown).

To investigate the differentiation of stem cells that had taken place in vivo before sacrificing the animals, immunofluorescence for expression of cardiac markers was performed on cardiac slides. Primary antibodies used were mouse antibodies against desmin (1:25, Sigma), Troponin T (1:25, AbD Serotec, Oxford, UK) or MLC-2α (1:25, Synaptic Systems, Gottingen, Germany). Secondary antibody used was Cy-5 labeled goat-anti-mouse antibody (1:100, 30 min, Molecular Probes). Slides were covered with mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA, USA).

To analyze the number of stem cells in the infarcted area of the heart, five random images in the infarcted area (magnification 100×) were analyzed per slide (3 slides per rat). Pictures were taken of the infarcted area, in such a way that always lets at least a small border of healthy myocardium

Table 1 Population doubling time for rat ASCs in culture. (n=3).

Passage	Population doubling time (days)
1	1.87±0.10
2	2.46±0.40
3	2.38±0.28
4	2.59±0.22

be visible. Cells were recognized via their red fluorescence, since before injection cells were labeled with the fluorescent lipid DiI. As erythrocytes show high auto-fluorescence, false positive cells were excluded by scoring only those cells as real stem cells with a visible nucleus (DAPI staining).

Statistical analysis

Statistical analysis was performed using GraphPad Prism 4 software. A t-test or ANOVA was used if scores were distributed normally, if not, a Mann–Whitney test or Kruskall–Wallis test was used for analysis. A Pearson chi-square test was used for analysis of discrete values. A p-value smaller than 0.05 was considered to be statistically significant. In the text and figures values are given as mean±standard error.

Results

Characterization of rat stromal vascular fraction and adipose derived stem cells

From each rat approximately 10 g of fat was resected, from which approximately 10^7 nucleated cells were isolated. Colony forming unit assays showed that these SVF cells contained 4.4±0.3% (n=4) cells that were able to form a colony. The population doubling time of ASCs increased from 1.9 days at passage 1 to 2.6 days at passage 4 (Table 1). Cells remained 14±1 days in culture, which corresponds with 6–7 population doublings, before transplantation after passage 2.

Further analysis of the characteristics of the rat SVF cells was performed using FACS analysis. We then found that the SVF fraction was a heterogeneous mixture of cells (n=3), containing populations positive for the stem cell markers CD34, CD73, CD90, and CD105, but also populations with markers for other cells like leukocytes (CD45) and endothelial cells (CD31). It was further found that during culture, cells became more homogeneous. The percentage of cells positive for the stem cell markers namely increased during culture, while markers for other cell populations like leukocytes (CD45) and endothelial cells (CD31) decreased (Table 2). In addition, SVF cells and cultured ASCs showed aldehyde dehydrogenase activity (not shown), which also is a characteristic for stem cells (Moreb, 2008). These data are comparable to those we have shown before for human SVF and cultured ASCs (Oedayrajsingh-Varma et al., 2006). It was further found, using the forward scatter in FACS analysis, that the cell size of the cultured ASCs increased when compared to fresh SVF cells (Fig. 1A). When analyzing exact cell size it was indeed found that the mean diameter of passage 2 ASCs was significantly larger than that of SVF cells (20.3±0.69 μm for cultured ASCs versus 11.3±0.24 μm for fresh SVF cells, $p<0.01$, n=3, Fig. 1B).

SVF administration reduced infarct size in a rat model of AMI

To analyze the putative effect of stem cell therapy on the infarction area in a reperfusion model of AMI in immunocompetent rats we intravenously injected uncultured SVF cells and cultured ASCs, 7 days after AMI. It was found using histological analysis at 35 days after AMI, that the hearts showed scar tissue formation, mainly composed of fibrosis with some inflammatory cells (mainly macrophages), and also contained blood vessels (Figs. 2A, B). No differences were found in scar composition between control rats and rats receiving stem cells (SVF cells or ASCs). We neither found differences in wall thickness between groups receiving stem cells nor vehicle (anterior wall vehicle 1.55±0.06 mm, SVF 1.65±0.09 mm, ASC 1.51±0.04 mm), using echocardiography analysis 4 weeks after transplantation. However, significant differences were found in scar size. Both treatment with SVF and ASC reduced infarct size with respectively 51±10% (n=12) and 58±12% (n=8), when compared to vehicle (n=11, $P<0.05$ both, Fig. 2C). Infarct size, however, was not significantly different between ASC- and SVF-treated rats ($p=0.69$).

SVF administration improved cardiac function after AMI

Heart function was analyzed using echocardiography at baseline, before transplantation on day 7, and before the

Table 2 Expression of different CD-markers on SVF cells and passages 1–4 ASCs. Numbers are shown as percentage of positive cells. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ (n=3).

	SVF	Passage 1	Passage 2	Passage 3	Passage 4
CD31	20.3±2.5	2.4±0.3**	0.5±0.1**	0.2±0.0**	1.0±0.3**
CD34	28.1±2.3	4.6±1.0**	1.8±0.1**	0.7±0.4**	0.8±0.2**
CD45	48.4±1.3	8.1±1.7**	3.6±0.3**	2.7±0.2**	2.4±0.4**
CD54	12.9±3.8	5.6±1.0	5.9±0.2	4.3±1.7	5.7±0.9
CD73	64.3±1.5	99.0±0.4**	97.9±1.1**	92.2±0.6**	92.2±1.2**
CD90	56.2±0.4	99.9±0.0**	100.0±0.0**	99.2±0.1**	99.2±0.4**
CD105	69.2±2.1	99.3±0.4**	99.8±0.0**	46.8±7.1**	51.6±6.6*
CD166	14.0±4.4	17.4±5.1	3.8±2.0	3.2±1.2	6.7±2.3

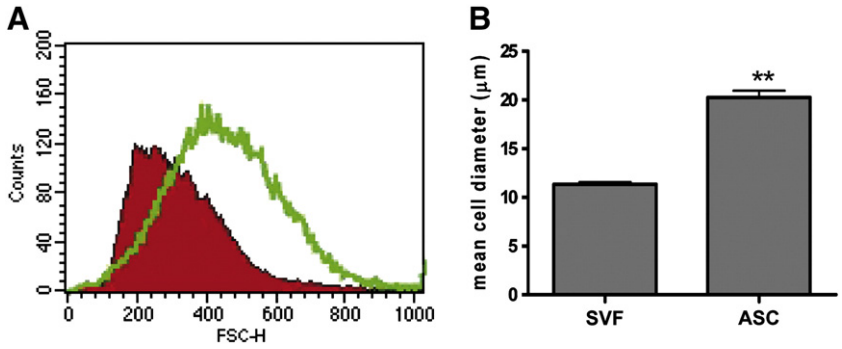

Figure 1 Cultured ASCs are larger than uncultured SVF cells. A) FACS plot of forward scatter, representing cell size, of fresh SVF cells (red plot), and cultured ASCs (green line). Cultured ASCs are larger than uncultured cells (mean in arbitrary units 445 ± 149 for cultured ASCs versus 318 ± 125 for fresh SVF cells). B) Average mean cell diameter (n = 3) for SVF and passage 2 cultured ASCs. ** $P<0.01$.

rats were sacrificed on day 35. To correct for inter-individual variance, cardiac function on day 35 was expressed as percentage of function at day 7 (100%) before transplantation of the same animal (Fig. 3). Although cultured ASCs improved fractional shortening after AMI, only a trend of improvement was found after treatment with SVF cells ($p<0.1$), when compared to vehicle-treated rats (Fig. 3A). Furthermore both SVF and cultured ASC therapy improved stroke volume back to baseline level, although this also was not statistically significant ($p<0.1$, Fig. 3B). Taken together SVF treatment reduced infarct size and improved heart function in a similar way that ASC treatment did.

Stem cell retrieval

At day 35 after AMI, we analyzed the number of injected cells that could be retrieved from the infarcted areas of the heart. Labeled cells were found as solitary cells, only in the infarcted area of the heart, mainly near the border zone, and not in the non-infarcted part of the heart, nor in other organs (not shown). In the SVF cell treated group, 13 ± 5 cells/mm^2 and in the cultured ASC treated group, 11 ± 3 cells/mm^2 were detected in the infarcted area, 35 days after AMI.

To evaluate whether the injected cells had differentiated into cardiomyocytes, serial heart sections were stained for the cardiomyocyte markers MLC-2α (Figs. 4C–D), desmin (Figs. 4F–G), and Troponin T (Figs. 4I–J). In Fig. 4, cardiac markers are shown in green, stem cells in red, and nuclei in blue. All identified stem cells (both from fresh SVF cells and cultured ASCs) were positive for these cardiac specific markers (Fig. 4). However, no retrieved stem cells were found to be positive for endothelial cell markers (CD31, CD146: not shown). It needs to be noticed that the stem cells in the heart of the ASC rat that died shortly after transplantation were negative for cardiac markers (not shown). This suggests that cells indeed had differentiated towards cardiomyocytes in vivo, since cells were negative for markers at the time of injection.

Vascularization in the infarction area

At day 35 after AMI, we analyzed the total number of blood vessels and the number of arterioles in the total infarcted area and its border zone using CD31 and SMA staining respectively. Using CD31 staining we found that injection of SVF (both at 1 or 7 days after AMI) did not significantly increase the total number of vessels in the infarcted area, when compared to the untreated control (control: 877 ± 111 vessels/mm^2, SVF 1 day: 764 ± 145 vessels/mm^2 $p=0.54$, SVF 7 days: 1067 ± 161 vessels/mm^2, $p=0.38$). In the ASC group

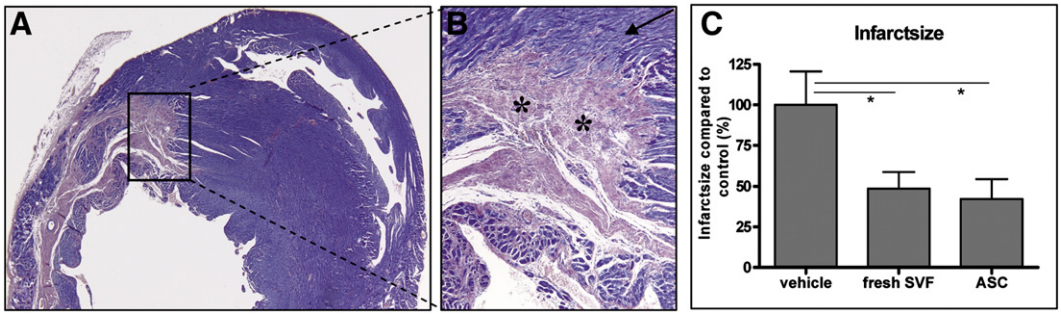

Figure 2 Treatment with stem cells reduced infarct size. A+B) PTAH staining of myocardium with infarction (vital myocardium is stained blue/arrow, fibrosis red/asterisk). A) Magnification 50×, B) magnification 400×. C) Relative infarct size compared to the vehicle group (infarct size was decreased with 51 ± 10% in rats receiving SVF cells (n = 12, * $p<0.05$, ANOVA) and with 58 ± 12% in rats receiving ASCs (n = 8, $p<0.05$, ANOVA).

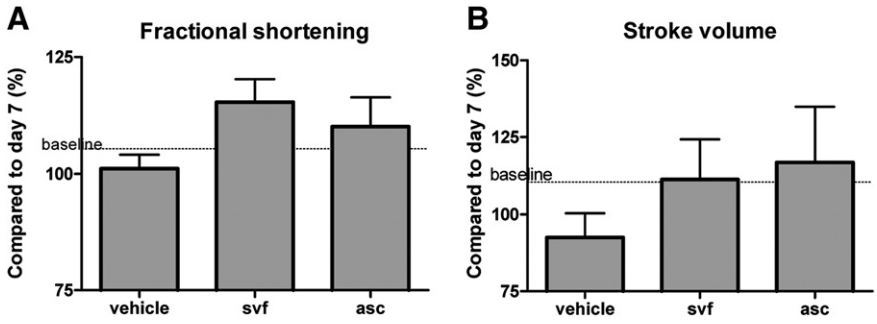

Figure 3 Treatment with stem cells improved cardiac function. Cardiac function before sacrifice was corrected for function before transplantation, thus at 7 days (100%). Baseline values before infarction is shown as dotted line. A) Fractional shortening. SVF cells, more than cultured ASCs, showed a trend towards improved fractional shortening after AMI, when compared to vehicle-treated rats ($p<0.1$, ANOVA). B) Stroke volume. The vehicle-treated group has a non-significant decreased stroke volume when compared to baseline levels. Furthermore, a trend was found that both SVF cells and ASCs improved stroke volume, back to baseline level, although this was not statistically significant.

the number of vessels was increased even more when compared with the vehicle group, up to 1383 ± 284 vessels/mm^2, however this difference was borderline significant ($p<0.1$, Fig. 5A). The number of blood vessels in the ASC group was not significantly higher than in the SVF (7 days) group ($p=0.36$). In the border zone around the infarction area, no significant differences were found in the number of blood vessels between any of the groups (Fig. 5B).

Using SMA staining an increase in the number of arterioles was found in the infarcted area of the SVF group (both 1 day and 7 days post AMI), when compared to the untreated control. (Control: 30.6 ± 4.4 vessels/mm^2, SVF 1 day: 45.1 ± 10 vessels/mm^2 $p=0.13$, SVF 7 days: 47.2 ± 12 vessels/mm^2, $p=0.14$). However this again was not significant. In the ASC group the number of arterioles was increased compared with the vehicle group, up to 52.6 ± 8.4 vessels/mm^2, although this difference was only borderline significant ($p<0.1$, Fig. 5C). No significant differences were found between the different treatment groups. In the border zone around the infarcted area no significant differences in the number of arterioles between any of the groups were found (Fig. 5D).

Numbers of macrophages in the infarction area

In the vehicle-treated group 326 ± 110 macrophages/mm^2 were found in the infarcted area, in contrast to 235 ± 139 macrophages/mm^2 after SVF treatment, and 277 ± 151 macrophages/mm^2 after ASC treatment. This decrease in the number of macrophages by SVF cell and ASC treatment was not statistically significant when compared to vehicle ($p=0.1$ and $p=0.43$ respectively, Fig. 5E), suggesting that stem cell therapy did not affect macrophage infiltration.

Complications after intravenous injection

Although intravenous injection of both SVF and ASC resulted in a large and significant infarct reduction, it has to be pointed out that in the ASC group three animals showed severe shortness of breath directly following injection of ASC. One of the three died within several minutes after treatment due to pulmonary embolism (Fig. 6) while the other two animals survived. After 2 days of illness and shortness of breath, the other 2 animals recovered completely. In contrast, in the SVF-treated group, no complications were found during injection or thereafter.

Time point of transplantation

To study our hypothesis that SVF/stem cell application after 7 days would be more successful compared with 1 day post AMI, we analyzed the effect of application of SVF cells 1 day post AMI. As cultured ASC transplantation resulted in complication in these rats, we preferred to limit this part of the study to SVF cells only.

We found that SVF cells applied 1 day after infarction, did reduce scar size by $30 \pm 20\%$ ($p=0.35$). This reduction was clearly decreased compared with the reduction after transplantation 7 days post AMI ($51 \pm 10\%$), and was not statistically significant when compared to vehicle.

Discussion

In this study we investigated in an immunocompetent rat model of AMI with reperfusion, whether intravenous injection of uncultured SVF or cultured ASCs 7 days after AMI, improved AMI outcome, and finally compared this with injection of SVF cells 1 day after AMI. It was found that intravenous delivery of stem cells (both SVF and cultured ASCs) 7 days after AMI significantly reduced infarct size, and improved cardiac function. We further found that stem cells could be detected in the infarcted area and had differentiated towards cardiomyocytes, although the amount of retrieved cells was limited. Stem cell therapy did not significantly improve vascular density, however we found a trend towards an increased number of arterioles and total vessels in the infarcted area, but not in the border zone, after treatment with SVF cells and cultured ASCs. The number of macrophages in the infarcted area was not significantly affected by cell therapy. No adverse effects were found after injection of SVF, whereas a detrimental

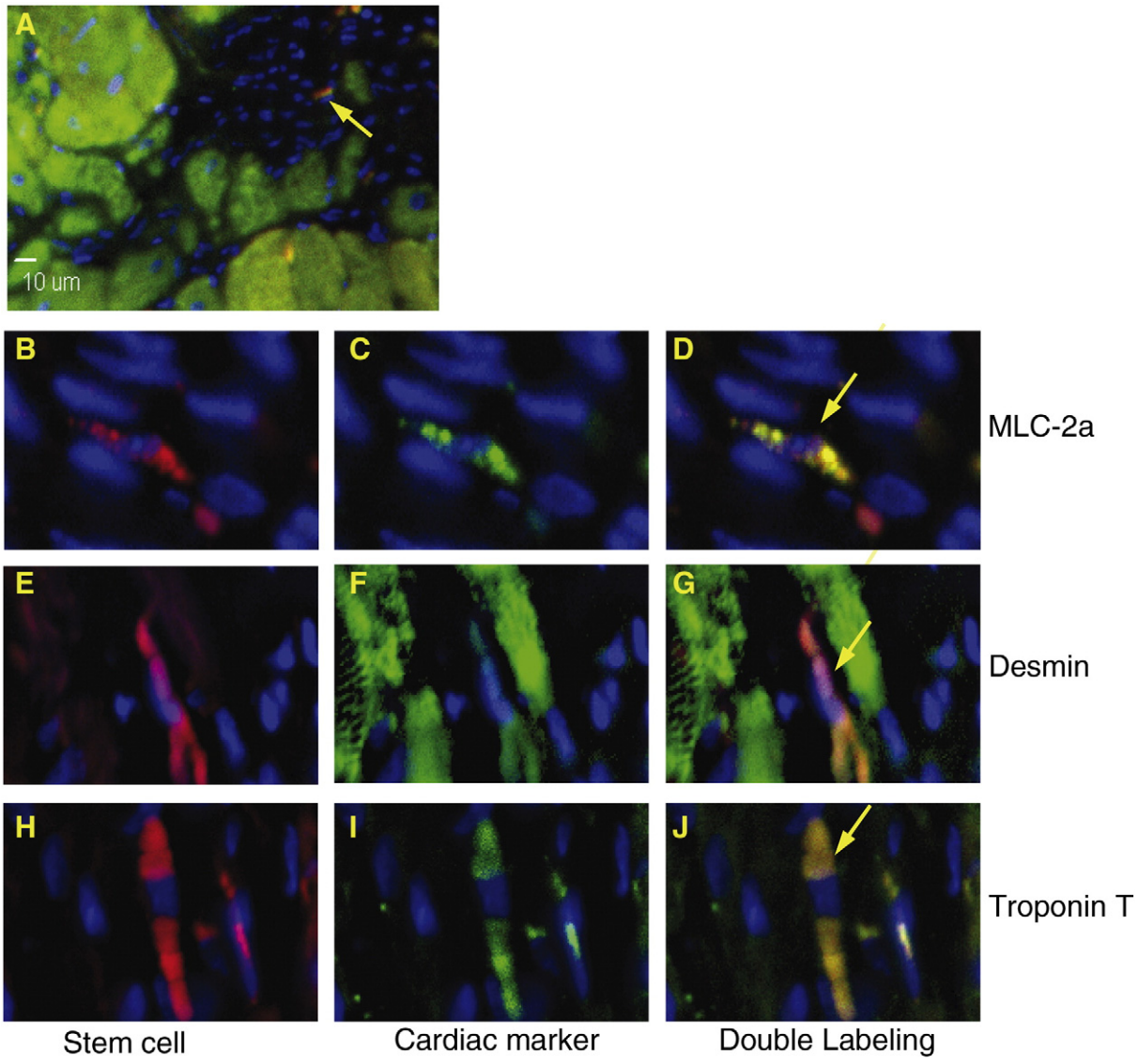

Figure 4 Differentiation of ASCs towards cardiomyocytes. Immonufluorescence staining for cardiac markers. A) Overview of the border zone of infarction, showing healthy cardiomyocytes on the left (green), and infarcted area on the upper right side. B, E, H) Stem cells are detected in the infarcted area (red, arrow). C, D) Myosin Light Chain-2a, F, G) desmin, and I, J) Troponin T. Differentiation was found both for SVF and cultured ASCs towards cardiomyocytes. Cardiac marker = green, nucleus = blue, DiI labeled cells = red. Merged images (D, G, J) show double stained cells (arrows), thus stem cells that differentiate towards cardiomyocytes (red + green = orange). Magnification: 100×.

effect on respiratory function was found in three animals in which cultured ASCs were used. Finally, it was found that in contrast to injection 7 days after AMI, injection of SVF cells 1 day after AMI, only resulted in a small non-significant infarct reduction.

We herewith have shown that freshly isolated SVF improve cardiac outcome comparable to cultured ASCs in an immunocompetent rat model of AMI with reperfusion. Our data are comparable to data of Bai et al., who have also shown that both fresh SVF cells and cultured ASCs improved cardiac function in a comparable way (Bai et al., 2010). However, we now have additionally shown that these stem cells truly reduced scar size, which was not analyzed by Bai et al. directly. In addition, we have applied intravenous delivery of these cells, which makes the application of this technique in patients easier, compared to intramyocardial injection. Notwithstanding, both studies demonstrate that these mesenchymal stem cells truly have the potential of homing to the infarcted heart. In addition we have used fully immunocompetent rats, which was possible since we used cells from syngeneic animals, which is more comparable to a clinical setting than the immunodeficient mice Bai et al. have used in their study. In contrast to the study of Bai et al., we did find one important difference between injection of fresh SVF cells and cultured ASCs, namely that in the ASC group three animals showed severe shortness of breath directly following injection of ASC, and one of these animals died within several minutes after treatment, due to thrombo-emboli of the lung, which was not found in the SVF-treated group.

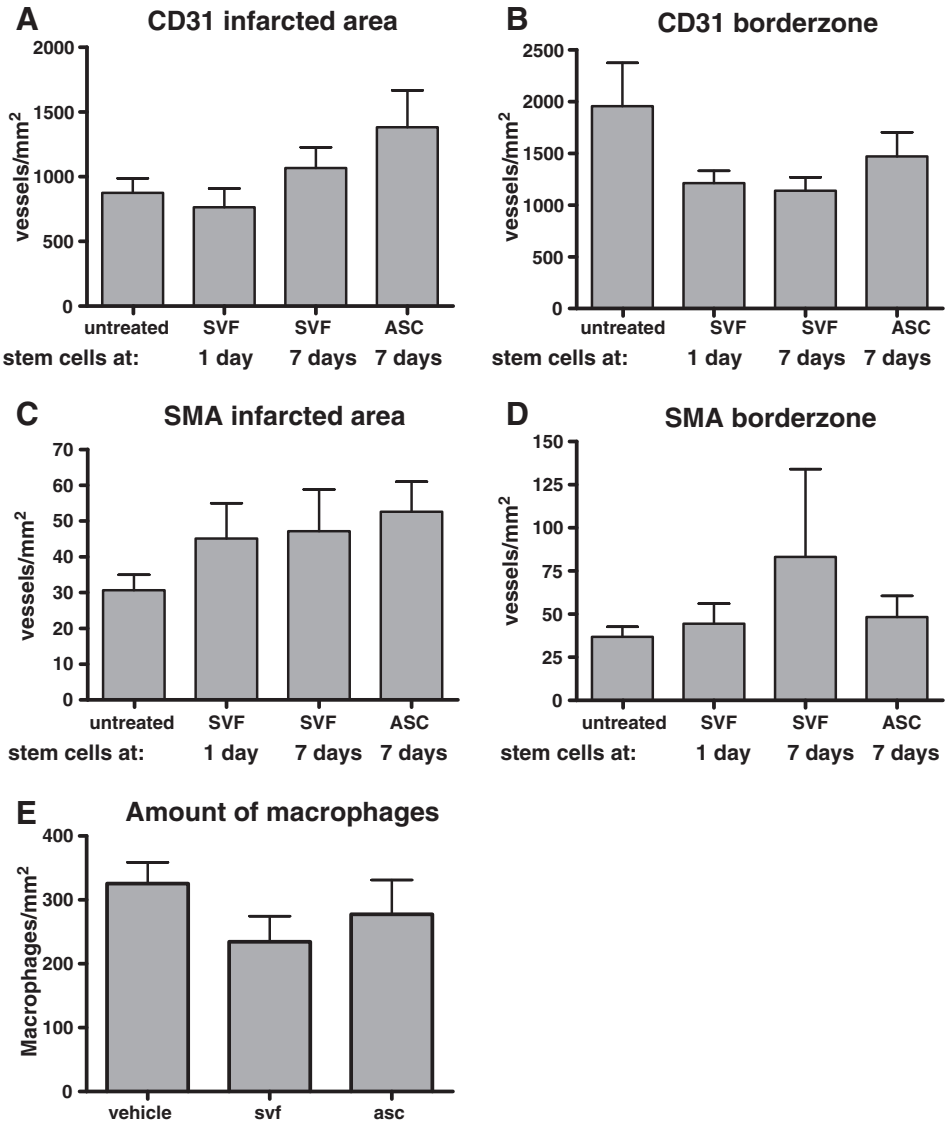

Figure 5 Number of blood vessels and macrophages in the infarcted area and border zone. A+B) Numbers of CD31 positive vessels in the infarcted area and the border zone respectively. C+D) Numbers of SMA positive vessels in the infarcted area and the border zone respectively. E) Numbers of macrophages were reduced both in the SVF group and the ASC group the number, however this was not statistically significant, although a trend was shown for decreased macrophage numbers in the SVF group ($p<0.1$, when compared to vehicle, ANOVA).

This detrimental effect of cultured ASC on the pulmonary circulation was probably caused by the entrapment of ASCs in the lungs, related to increased size of ASCs due to the culturing procedure, also shown in this study. It indeed has been described previously that culturing of stem cells increases cell size and also increases the expression of adhesion molecules, which may lead to complications during transplantation (McIntosh et al., 2006; Zhu et al., 2008). Furthermore, it is known that respiratory failure is the most common complication after bone marrow transplant (Gao et al., 2001). Since the results in cardiac outcome between fresh SVF cells and cultured ASCs is comparable, and culturing is time consuming, expensive, and leads to more complications, our study seems to indicate that the use of SVF cells is clinically more favorable.

In this study we also found a trend towards improved stroke volume and fractional shortening after stem cell treatment, compared to the vehicle-treated group, although this was not statistically significant. However, it should be pointed out that infarct size was relatively small. We however deliberately induced non-aneurysmatic infarcts by placing the suture approximately 2 mm below the origin of the coronary artery, since in patients most infarcts are non-aneurysmatic, because of the widespread use of immediate reperfusion therapy. This might explain its small effect on heart function after AMI and in turn also the small effect of stem cell treatment on the reduced function (Ndrepepa et al., 2007; Pfeffer et al., 1979; Schaer et al., 1990).

Another important finding of this study is that stem cells were detected in the infarcted area 28 days after AMI, using

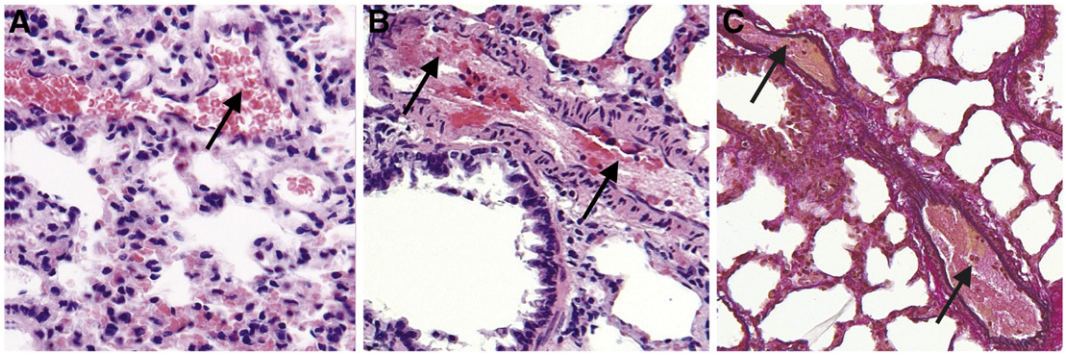

Figure 6 Thrombo-embolism after cultured ASC injection. A) HE staining of a lung of a rat treated with ASC that was sacrificed at 35 days after infarction, showing erythrocytes in the vessels (arrows), without thrombo-embolism. B) HE staining of a lung of a rat treated with cultured ASC that died shortly after injection of cells, showing thrombo-embolism including fibrin fibers in blood vessels (arrows). C) EVG staining of the lung from the same rat as B, showing thrombo-embolism, including infiltration of inflammatory cells (arrows). Original magnification 100×.

CM-DiI labeling, a label that is not exchanged between cells, and can be traced up to 6 weeks in vivo, also after fixation (Andrade et al., 1996; Weir et al., 2008). Even more importantly, we found that all detected stem cells showed cardiomyogenic differentiation. However, the number of detected stem cells was relatively low, suggesting that the improvement of infarction is most likely not explained by any differentiation of ASCs into cardiomyocytes.

Reduction in infarct size might partly be explained by improved angiogenesis (Mazo et al., 2008; Miyahara et al., 2006; Zhang et al., 2007). We indeed did find trends of increased numbers of total vessels and arterioles in the infarction area after treatment with stem cells, although this was borderline statistically significant. Several other studies have described in small animal models that the effect of ASC transplantation after AMI can be explained by differentiation towards endothelial cells (Mazo et al., 2008; Miyahara et al., 2006; Zhang et al., 2007). However, we did not find differentiation of SVF cells or ASCs towards endothelial cells. Strem et al. (2005) also only found differentiation of stem cells towards cardiomyocytes, but no differentiation towards endothelial cells, in a mouse model in which SVF cells were transplanted directly after reperfusion (Strem et al., 2005). Differences in studies might be explained by the time point of injection, resulting in altered differentiation signals for the cells. These studies namely transplanted ASCs late after infarction (>3 weeks). While both in the study of Strem et al. and in our study, cells were transplanted within 1 week after infarction.

Another pathway by which ASCs might improve cardiac outcome is by a paracrine effect, whereby theoretically growth factors, cytokines and signaling molecules produced by the infused stem cells reduce inflammation, and favor the viability of myocytes by inhibiting apoptosis signaling (Uemura et al., 2006). This has already been suggested for embryonal stem cells and bone marrow MSC (Guo, 2007; Singla, 2007). In addition, it has been described that ASCs prevented apoptosis of cardiomyocytes subjected to hypoxia in vitro, through excretion of IGF and VEGF (Sadat et al., 2007). The cardiac improvement we have found after stem cell injection in our rat model of AMI, might be explained by this phenomenon.

Another important finding is that, to the best of our knowledge, this is the first study that shows that intravenous injection of SVF cells reduced myocardial infarction size. A commonly used clinical transplantation method is to inject stem cells during percutaneous coronary intervention. However this option would not be clinically practical if stem cell delivery were to take place after the acute inflammation phase after AMI, as in the present study. Intravenous delivery is the least invasive injection method, and also provides an easy possibility to inject more than a single bolus. 0.09pt?>Therefore intravenous injection is clinically most favorable. However, intravenous administration requires that the stem cells home actively towards the infarcted area. Indeed we could only detect stem cells in the infarcted area in the heart 35 days after AMI, and not in other organs, suggesting that stem cells did home specifically to the injured heart. Similar results have been described by pig and rat studies using different types of stem cells (Min et al., 2006; Wolf et al., 2009).

Finally, we investigated the ideal time point for stem cell delivery. We did find that application of stem cells 7 days post AMI is preferable over 1 day, since at 7 days scar size was decreased by 51%, compared to only 30% reduction when SVF was injected at day 1. This difference is probably caused by the difference in the environment in the heart after AMI. During the acute phase of infarction massive myocardial necrosis and leukocyte infiltration theoretically may harm survival of implanted cells (Azarnoush, 2005; Wang and Sjoquist, 2006). Our results are comparable to other studies that investigated the timeframe after AMI when to apply stem cell therapy, both in animal models and human stem cell transplantation studies, by investigating molecular events and stem cell retention at the site of infarction (Azarnoush, 2005; Bermejo et al., 2006; Lu et al., 2004; Ma, 2005; van Dijk et al., 2008a, 2008b) These studies suggest that the optimal timing for stem cell therapy is after the acute inflammation period (over 1 day after infarction), and before 2 weeks after AMI when scar tissue has been formed. Indeed, Jiang et al. (Jiang et al., 2008) also found that 1 week after AMI was the best time point for delivery of bone marrow MSCs to improve cardiac outcome after AMI (Jiang et al., 2008).

However up to now, no time point delivery studies have been performed for SVF cells or cultured ASCs.

In conclusion, we now show for the first time that intravenous injection of both fresh SVF cells and cultured ASCs cells 7 days after AMI, significantly reduced myocardial infarction size in an immunocompetent rat AMI model with reperfusion. In addition, injection of SVF cells did not lead to complication, in contrast to injection of ASCs. Therefore, SVF transplantation has a high clinical potential, since these cells can be harvested easily, and injected intravenously without risk of complications.

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