Bone marrow–derived mononuclear cell transplantation improves myocardial recovery by enhancing cellular recruitment and differentiation at the infarction site

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Objective: Stem cell therapy in myocardial infarction is under intensive investigation; however, the mechanisms of recovery and the optimal transplantation technique remain controversial. The goal of this controlled and randomized study was to test the hypothesis that locally injected bone marrow–derived mononuclear cells can focus in on the damaged myocardium and improve cardiac function by means of active participation in remodeling.

Methods: Myocardial infarction was introduced through occlusion of the circumflex coronary artery for 90 minutes in 14 piglets (24.0 ± 4.9 kg) that were randomized to a cell-therapy group ($n = 7$) and a control group ($n = 7$). At reperfusion, autologous purified prelabeled or unlabeled cells (10^8 cells/2 mL) or saline were injected into the myocardium. Cardiac function was measured by using echocardiography preoperatively and postoperatively and at 3 weeks, when hearts were collected for histopathologic examination.

Results: The ejection fraction recovered in the cell-therapy group ($P = .02$) but failed to recover in the control group, and at 3 weeks, it remained at the lower level compared with that in the cell-therapy group ($P = .067$). The number of living cells in the necrotic area was significantly greater in the cell-therapy group ($P < .001$). Labeled cells were detected in the infarcted area, and they showed signs of myocyte differentiation. Furthermore, the proportional area of muscle actin-positive cells at the granulation area was higher in the cell-therapy group ($P = .035$).

Conclusions: Autologous bone marrow–derived mononuclear cells at the infarcted area localize in the myocardium. The exact mechanism of recovery remains to be determined, but our findings may give new information concerning the cellular events that occur during cell therapy–enhanced recovery.

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Understanding the functions of stem cells and learning to control them will open new avenues for the treatment of many diseases. Knowledge about the role of stem cells in myocardial recovery has increased substantially, but many details of the mechanisms of cell differentiation and myocardium recovery are still under investigation.

Abbreviations and Acronyms

α -SMA	= α -smooth muscle actin
BMMC	= bone marrow–derived mononuclear cell
EF	= ejection fraction
IQR	= interquartile range
MSA	= muscle-specific actin

It is known that stem cell transplantation to the damaged myocardium improves recovery significantly compared with the normal injury-repairing processes occurring in the myocardium. Histopathologic data from preclinical studies have confirmed the recovery of the infarcted area.¹⁻³ Furthermore, it is known that stem cells can differentiate toward cardiomyocytes.⁴⁻⁷ It has been shown that the stem cells release growth factors and signals in the myocardium that participate in tissue-repairing processes.⁸⁻¹⁰

Stem cell therapy in myocardial infarction is under intensive investigation, although it has already been adopted in clinical use. Most studies are based on the intracoronary transplantation method, and the intramyocardial method has been used in only a few studies. These two transplantation methods seem to be the most effective, safe, and administrable in clinical use. Perhaps the bone marrow–derived mononuclear cells (BMMCs) are not the ideal type of cells, but because of the lack of immunologic rejection and with easy access and purification, they are clinically relevant, as recent studies have indicated. In addition, the use of autologous BMMCs does not raise ethical issues in clinical application. The outcomes in clinical studies fluctuate from moderate to substantial recovery of the myocardium.¹¹⁻¹⁶

Positive preclinical results have shifted the focus of research into clinical studies, despite the unknown functional mechanisms of the stem cells. We find our experimental porcine infarction model relevant in providing new information for stem cell research.

We established a porcine acute myocardial infarction model, which mimics open coronary artery bypass grafting surgery, to evaluate the efficacy of delivering bone marrow–derived cells by means of direct injection into the damaged myocardium. The hypothesis in our experimental design was that BMMC transplantation immediately after infarction recovers the function of the heart after 3 weeks. Furthermore, we wanted to clarify whether a direct injection into the infarcted area and into the border zone intensifies homing of the stem cells and accelerates repair processes.

Materials and Methods**Preoperative Management**

Twenty-six juvenile pigs (8–10 weeks of age) from a native stock were operated on in a sterile environment. The first 6 animals underwent feasibility experiments to optimize logistics and cell-

purifying techniques. Of the remaining 20 animals, 6 pigs died during coronary occlusion because of ventricular fibrillation. These animals were excluded from further analysis, and the deaths were not linked to cell or vehicle injection. Fourteen pigs were randomized to the BMMC group ($n = 7$) and a control group ($n = 7$). The median weight of the pigs was 23.6 kg (interquartile range [IQR], 21.7–27.3 kg) in the cell-therapy group and 24.5 kg (IQR, 19.5–27.6 kg) in the control group. All animals received humane care in accordance with the “Principles of laboratory animal care” formulated by the National Society for Medical Research and the “Guide for the care and use of laboratory animals” prepared by the Institute of Laboratory Animal Resources, National Research Council (published by the National Academy Press, revised in 1996). The study was approved by the Research Animal Care and Use Committee of the University of Oulu.

Anesthesia

The animals were sedated with an intramuscular injection of ketamine hydrochloride (350 mg) and midazolam (45 mg). A peripheral (venous) catheter was inserted into a vein of the right ear for administration of drugs and to maintain fluid balance with Ringer acetate. After an induction with fentanyl (25 μ g/kg administered intravenously) and pancuronium (0.2 mg/kg administered intravenously), the pigs were intubated, and the balanced anesthesia was maintained with a continuous infusion of fentanyl (25 μ g $\cdot$ kg⁻¹ $\cdot$ h⁻¹), midazolam (0.25 mg $\cdot$ kg⁻¹ $\cdot$ h⁻¹), and pancuronium (0.2 mg $\cdot$ kg⁻¹ $\cdot$ h⁻¹) and inhaled isoflurane (0.5%) in both study groups throughout the whole experiment. The animals were maintained on positive-pressure ventilation with 50% oxygen. Cefuroxime (1.5 g administered intravenously) was administered at anesthesia induction and before extubation.

Isolation of Mononuclear Cells

The animals were placed in a supine position, and 2 $\times$ 10 mL of bone marrow was aspirated beneath the tibial tuberosity into a syringe containing 5000 IU of heparin. The bone marrow aspirates were delivered for isolation to the laboratory situated within the same facility as the operating room. The bone marrow aspirate (dilution 1:5 in phosphate-buffered saline, Sigma-Aldrich) was passed through a density-gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences) to exclude erythrocytes and granulocytes. Mononuclear cells were collected from the interphase and washed 3 times with phosphate-buffered saline. The cells were then suspended in a saline solution at a density of 50 $\times$ 10⁶ cells/mL. Trypan blue dye was used to stain a fraction of isolated cells to confirm the viability of more than 99% transplanted cells. For the homing experiment, the isolated mononuclear cells were labeled with Vybrant CM-DiI (Molecular Probes), according to the manufacturer’s instructions.

Hemodynamic and Cardiac Function Monitoring

Transthoracic echocardiography was performed with the Acuson Sequoia 512 echocardiography system with a 3.5-MHz transducer (Siemens) preoperatively and at 30 minutes and 3 weeks after instituting reflow. The echocardiography was performed blinded to the treatment group. The thicknesses of the ventricular walls (left ventricular posterior wall diameter, interventricular septum diameter, left ventricular end-systolic diameter, and left ventricular

end-diastolic diameter) were measured from the M-mode view of the left ventricle. The ejection fraction (EF) was calculated by using the Teichholz formula, and the left ventricular mass was calculated by using the method described by Reichek and Devereux.^{17,18} Transmitral flow peaks E and A and their ratio were measured from the 4-chamber view with the pulsed-wave Doppler sample volume between mitral leaflet tips, and the isovolumetric relaxation time was calculated from the aortic valve with continuous-wave Doppler scanning. Cardiac output was measured by using the velocity-time integral of the pulsed-wave Doppler sample from the left outflow tract, the diameter of the left outflow tract, and the heart rate.

An arterial catheter was positioned in the right femoral artery for arterial pressure monitoring and blood sampling. A thermodilution catheter (Criticath, 7 F; Ohmeda GmbH & Co) was placed through the right femoral vein to allow sampling of blood; monitoring of central venous pressure, pulmonary artery wedge pressure, and pulmonary capillary wedge pressure; and recording of blood temperature and cardiac output. During the occlusion of the circumflex coronary artery, the hemodynamic parameters were measured every 15 minutes.

Biochemical Analysis

Blood gases, pH, electrolytes, serum ionized calcium levels, glucose levels, hemoglobin levels (i-STAT analyzer, i-STAT Corp), basic blood count, leukocyte differential count (Cell-Dyn analyzer, Abbott), and creatine kinase MB (Hydrasys LC-electrophoresis, Hyrys-densitometry; Sebia) and troponin-I levels were measured at baseline, at 90 minutes after the infarction, and at 30 minutes and 24 hours after initiation of reperfusion.

Myocardial Infarction and BMBC Transplantation

The pericardium was opened through the left anterolateral thoracotomy in the fourth intercostal space, and the heart was exposed. The baseline parameters were recorded. The circumflex coronary artery was dissected just proximal to the left anterior descending coronary artery. Myocardial infarction was instituted by occluding the circumflex coronary artery with a removable silicone vascular loop (Surg-I-Loop, Scanlan) for 90 minutes. The ligation was done by the cardiac surgeon, who was unaware of the experimental design and the fate of the individual animals. At the end of the ischemic period, the silicone loop was removed, and 1 mL of papaverine was administered on the vessel to avoid spasm; reperfusion was confirmed visually. Immediately after the initiation of reperfusion, autologous purified cells (10^8 cells/2 mL) or saline (2 mL) were transplanted by means of intramyocardial injection into the infarcted area. Solution was administered carefully with a needle (26-gauge) attached to a syringe to 10 different locations in and around the infarcted area. Prolene sutures (4-0; Ethicon, Inc) were used to mark the infarcted area. A single pleural tube was used for draining, and the thoracic cavity was closed. The animals were extubated 2 hours after reperfusion. On the following day, 24 hours postoperatively, the animals were sedated, and blood samples were taken from the external jugular vein.

Histopathologic Examination

At the 21st postoperative day, the pigs were sedated. Blood samplings and measurements of hemodynamics and blood flow by

means of echocardiography were performed as previously described. Each surviving animal was electively killed with an intravenous injection of pentobarbital (60 mg/kg) and heparin (500 IU/kg), and the hearts were removed for histopathologic analysis. Ischemic and control tissue samples from the left ventricular wall were formalin fixed. The harvested tissues were paraffin embedded and sectioned at 5- μ m thickness for hematoxylin and eosin, Van Gieson, Von Kossa, and Masson trichrome staining. A single pathologist, who was unaware of the experimental design and the fate of the individual animals, screened the sections of the heart specimens of each animal. The assessment of the injured area included the following: (1) granulation tissue, (2) the border zone of the necrotic and fibrotic infarcted areas, and (3) the severely infarcted area (local necrosis). The quantity of living cells in the necrotic area was detected by using immunofluorescent Hoechst staining (Developmental Studies Hybridoma Bank). Similarly, immunofluorescent Stro-1 staining (Developmental Studies Hybridoma Bank) was used to detect stem cells in the necrotic area. Quantification of these 2 stained areas from the border zone and granulation tissue was totally prevented by the autofluorescence caused by the degenerating cardiomyocytes. Quantification was carried out by using a computer program (Scientific Image Analysis; MCID-M4 3.0 Rev 1.1). Quantification of the immunostainings muscle-specific actin (MSA; DakoCytomation), myofibroblast marker α -smooth muscle actin (α -SMA; DakoCytomation), proliferation marker Ki-67 (NovoCastra), and the mesenchymal cell marker vimentin (DakoCytomation) covered the whole necrotic area and surrounding border zone of the necrosis of the samples. Quantification of the granulation tissue was limited so that at least a double-sized area compared with the necrotic area

Figure 1. Bone marrow–derived mononuclear cell (BMMC) injection at the infarction site enables ejection fraction recovery. The ejection fraction decreased after infarction critically ($>15\%$) in both groups ($!P = .03$). After the 3-week recovery period, the ejection fraction increased significantly in the BMMC group ($*P = .02$) but remained at the same decreased level in the control group. Data points represent actual median values $\pm$ standard deviation.

TABLE 1. Group statistics: Echocardiography

	Protocol	N	Baseline	Postoperative	3 wk postoperative
LVEDD (mm)	BMMC group	7	29.3 (28.7–31.6)	30.2 (29.9–32.6)	37.6 (37.4–39.3)
	Control group	7	30.1 (28.8–38.8)	30.1 (28.9–35.3)	36.5 (34.5–43.4)
LVESD (mm)	BMMC group	7	19.1 (14.3–21.5)	22.1 (21.2–23.9)	21.6 (19.5–22.4)
	Control group	7	18.5 (14.4–22.4)	22.0 (19.9–22.9)	22.8 (22.0–30.9)
E _{max} (cm/s)	BMMC group	7	63.3 (57.9–67.0)	46.8 (37.5–53.3)	61.6 (54.5–93.3)
	Control group	7	69.9 (57.3–77.9)	58.0 (50.0–74.9)	67.4 (58.3–90.4)
A _{max} (cm/s)	BMMC group	7	40.8 (32.3–53.9)	39.1 (35.8–43.3)	52.5 (33.5–57.1)
	Control group	7	41.6 (32.5–46.0)	46.6 (42.5–53.6)	47.6 (39.6–86.7)
E/A	BMMC group	7	1.47 (1.23–1.65)	1.19 (0.83–1.38)	1.47 (1.29–1.65)
	Control group	7	1.58 (1.44–1.94)	1.18 (1.12–1.51)	1.14 (0.94–1.49)
IVRT (ms)	BMMC group	7	8.0 (8.0–8.7)	7.5 (5.3–7.6)	5.7 (5.4–6.4)
	Control group	7	8.0 (7.2–8.0)	7.5 (5.5–8.4)	8.5 (6.7–9.3)
Heart rate (beats/min)	BMMC group	7	95.0 (89.0–108.0)	115.0 (100.0–135.0)	115.0 (103.0–118.5)
	Control group	7	109.0 (103.0–113.0)	121.0 (105.0–129.0)	120.0 (99.5–131.0)
EF (%)	BMMC group	7	65.7 (61.5–78.5)	56.8 (49.8–61.7)	74.1 (72.3–78.4)*
	Control group	7	74.3 (71.9–77.4)	62.7 (52.0–67.2)	61.2 (55.9–64.7)

Echocardiographic data represent medians and interquartile ranges (25th–75th percentile). *LVEDD*, Left ventricular end-diastolic diameter; *LVESD*, left ventricular end-systolic diameter; *E_{max}*, maximal transmitral E-wave; *A_{max}*, maximal transmitral A-wave; *E/A*, transmitral flow peaks E and A and their ratio; *IVRT*, isovolumetric relaxation time; EF, ejection fraction. **P* = .02.

was quantified. That limitation was needed because of the huge size of the granulation tissue compared with the other areas. Likewise, variations of the infarct of each animal set its own limitations. The proportional area of cell coverage was counted from MSA, α -SMA, Ki-67, and vimentin stainings. The proportional area of the cell coverage was counted by using Van Gieson staining also to assess the amount of collagen in the granulation tissue. Stains were prepared with the immunoperoxidase method (EnVision).

For the cell-tracking analysis, 2 additional pigs received a transplantation of fluorescent DiI dye-labeled cells. After 3 weeks of follow-up, these animals were electively killed. Tissues from the liver, lung, spleen, and hilar lymph nodes and infarcted and normal tissue of the heart were sampled. Samples were paraffin embedded, sectioned, and prepared for analysis. DiI-labeled cells were detected from each sample by using a fluorescent microscope. Because these pigs did not conform to the original protocol, the results obtained were analyzed separately. Expression of α -SMA positivity in DiI-labeled cells was quantified from the granulation tissue area. For the analysis, granulation tissue was divided into 3 areas: (1) organized granulation tissue, (2) border zone, and (3) unorganized granulation tissue. Similarly, the percentage of α -SMA⁺-labeled cells from total α -SMA⁺ cells was calculated. Also, the percentage of α -SMA⁺-labeled cells attached to the vessel structures was determined.

Statistical Analysis

Statistical analysis was performed with the SPSS statistical program (SPSS version 14.0; SPSS, Inc, Chicago, Ill). Continuous and ordinal variables are expressed as the median with the IQR (25th–75th percentiles). Complete independence was assumed across animals (by random statement). The Mann–Whitney *U* test was used to assess the distribution of variables between study groups. Significance levels are reported for comparisons with the 2-tailed test.

Results

The groups did not differ significantly in any preoperative parameters. There were no significant differences between the groups preoperatively and postoperatively in infarction markers. In both groups a significant release of heart enzymes was observed postoperatively compared with baseline values, indicating that a critical-sized infarction had occurred. This was also confirmed by means of visual observation of the infarcted area during the operation. The mean weight of the animals increased in both groups in 3 weeks. The increase in the median weight was 11.5 kg (IQR, 10.8–14.8 kg) in the BMMC group and 8.4 kg (IQR, 6.4–9.2 kg) in the control group; the weight increase was higher in the BMMC group (*P* = .05).

Hemodynamic Analysis

The 90-minute occlusion of the circumflex coronary artery resulted in a significant reduction in EF compared with the baseline value (*P* = .03), and there was no difference between the groups. In the BMMC group EF decreased from 65.7% (IQR, 61.5%–78.5%) at baseline to 56.8% (IQR, 49.8%–61.7%); in the control group the decrease was from 74.3% (IQR, 71.9%–77.4%) to 62.7% (IQR, 51.3%–65.9%). After the 3-week recovery period, EF increased significantly in the BMMC group (56.8% [IQR, 49.8%–61.7%] vs 74.1% [IQR, 72.3%–78.4%], *P* = .02) but remained at the same decreased level in the control group (62.7% [IQR, 51.3%–65.9%] vs 62.4% [IQR, 55.9%–64.8%]). The increase in EF after the recovery period was higher after the BMMC injection (*P* = .067, Figure 1).

Figure 2. Injected Dil-labeled cells remain at the infarct site. Transplanted Dil-labeled (*white arrows*) cells were detected by using a fluorescent microscope (magnification $\times 20$) from the infarcted area (A) and from some of the perihilar lymph nodes (B). A sample from undamaged heart muscle was negative (C), as were samples from the liver (D), spleen (E), and lungs (F). Bars = 100 μm .

The left ventricular end-diastolic diameter increased similarly in both groups during the 3-week recovery period. The changes in the left ventricular mass and wall dimensions were similar in both groups. In diastolic parameters the transmitral flow peaks E and A and their ratio decreased after infarction in both groups and failed to recover in 3 weeks in both groups. The isovolumetric relaxation time did not change during the procedure (Table 1).

Histopathologic Analysis

All animals in the study groups had a large infarction, which was, as expected, located in the posterior wall of the left ventricle. Four of the 7 animals in both study groups had necrotic material in the center of the infarction area, and for those 4 + 4 animals, histologic analysis could be performed.

For the 2 animals that were not included in the study groups, DiI-labeled cells were transplanted in a similar manner, and these cells were detected in the injection/damaged area but not from the healthy area of the myocardium. Labeled cells were also found in the perihilar lymph nodes but not in any other organs sampled, such as the liver,

Figure 3. Injected Dil-labeled cells differentiate at the injection site. DiI-positive cells in the granulation tissue area at the infarcted myocardium showed positive expression of α -smooth muscle actin (α -SMA; A and B), Ki-67 (C and D; magnification $\times 20$), vimentin (E and F; magnification $\times 40$), and muscle-specific actin (MSA; G and H; magnification $\times 60$). Bars = 20 μm .

spleen, or lungs (Figure 2). The labeled cells that were observed in the infarcted myocardium showed positive expression of the proliferation marker Ki-67, the mesenchymal cell marker vimentin, α -SMA, and MSA, which was detected by immunostaining for these antigens. The DiI-labeled cells were found both in the necrotic area and the border zone; however, most of the labeled cells could be found in the area surrounding the severe infarction site (ie, in the active remodeling site; Figure 3). α -SMA⁺ DiI-labeled cells quantified from the organized granulation tissue area showed a median percentage of 94.4% (IQR, 92.3%–100%) of α -SMA positivity in DiI-labeled cells. Similarly, in the border zone of organized and unorganized granulation tissue, the median percentage of α -SMA⁺ DiI-labeled cells was 92.1% (IQR, 87.5%–100%). In the unor-

Figure 4. Injection of bone marrow-derived mononuclear cells (BMMC) enhances cell survival and recruitment at the infarction site parenchyma. Larger quantities of living cells in the necrotic area were observed with a fluorescent microscope (magnification $\times 20$) in the BMMC group by using Hoechst staining (A and B) compared with the control group (E and F). In cell counting a significant difference between the groups could readily be observed (I; *** $P < .001$). Similarly, a larger number of Stro-1-positive cells was detected in the BMMC group (C, D, and J; $P < .001$) compared with the control group (G and H). The highest density of Stro-1-positive cells was detected at the vessel walls in the BMMC group (D) in contrast to the control group (H). Bars in A, C, E, and G = 100 μm ; bars in B, D, F, and H = 20 μm .

ganized granulation tissue the median percentage of $\alpha\text{-SMA}^+$ DiI-labeled cells was 23.8% (IQR, 20.0%–25.0%). The percentage of $\alpha\text{-SMA}^+$ DiI-labeled cells from total $\alpha\text{-SMA}^+$ cells in the organized granulation tissue was 25.0% (IQR, 22.6%–31.6%). Likewise, in the border zone of the granulation tissues, the percentage was 27.5% (IQR, 25.4%–28.1%), and in the unorganized granulation tissue the percentage was 4.1% (IQR, 2.5%–5.1%). The median percentage of $\alpha\text{-SMA}^+$ DiI-labeled cells attached to the vessel structures in the organized granulation tissue was 14.3% (IQR, 11.1%–23.1%). In the border zone of the granulation tissues, the median percentage was 60.3% (IQR, 53.3%–87.5%), and in the unorganized granulation tissue the median percentage was 9.5% (IQR, 8.7%–13.3%).

The quantity of cells recruited in the necrotic area was calculated by using Hoechst staining, which showed a significantly larger quantity of living cells in the BMMC group ($P < .001$). Moreover, Stro-1-positive cells could be detected similarly ($P < .001$). In addition, Stro-1-positive cells accumulated in the vessel walls of the necrotic area (Figure 4). Notable is that Stro-1-positive cells were detected in larger quantities particularly in the border zone and in moderate quantities in the granulation tissue. However, quantification of these 2 stainings from the border zone and granulation tissue was totally prevented by the autofluorescence caused by the degenerating cardiomyocytes. For the histopathologic analysis, control biopsy specimens from the healthy areas of each heart were taken and stained with

Hoechst and Stro-1. Stro-1-positive cells were not found in those tissue samples.

A proportional area of cell coverage was counted from the immunohistologic samples by using vimentin, Ki-67, MSA, and $\alpha\text{-SMA}$ antibodies. The number of vimentin-positive mesenchymal cells was significantly higher in the border zone ($P < .001$) and in the necrotic area ($P = .013$) in the BMMC group. The proportional area of cell coverage for muscle actin staining was higher in the granulation tissue area in the BMMC group ($P = .035$, Figure 5). Smooth muscle actin staining showed no difference between the groups. Proliferation marker Ki-67 staining showed no differences between the groups in any sector of the defined infarction. Van Gieson staining was used to assess the collagen in the granulation tissue, and no difference was observed between the groups.

Discussion

The experimental model used in this study is based on a critical-sized myocardial infarction, and it mimics clinically a situation in which coronary occlusion results in a severe and permanent cardiac injury. A considerable decrease in EF and a significant release of heart enzymes were observed in every animal after the 90-minute occlusion of the circumflex coronary artery, confirming the severity of myocardial infarction. The injection of BMMCs into the myocardium ameliorated EF decrease in 3 weeks.

Figure 5. Injection of bone marrow-derived mononuclear cells (BMMC) promotes postinfarction remodeling. Analysis of the immunohistologic staining with a light microscope revealed differences between the control and BMMC groups (images with $\times 10$ magnification are shown). Enhanced vimentin staining (A and C), indicating a larger quantity of mesenchymal cells in the necrotic area (E; $P < .01$) and in the border zone (F; $P < .001$), could be seen in the BMMC group. For the muscle actin staining (B and D), the proportional area of cell coverage was greater in the granulation tissue area (G) in the BMMC group ($P = .035$). Bars = $50 \mu\text{m}$.

The principal finding of this study was that the number of mesenchymal cells in the border zone and in the necrotic area was higher after injection of BMMCs. This observation was based on a counted proportional area of the cell coverage. Moreover, the quantity of viable cells (Hoechst staining) in the necrotic area of the infarct was significantly larger in the BMMC group. In addition, the accumulation of the Stro-1-positive cells could be found in the vessel walls in the necrotic area. These findings indicate that the border zone and the necrotic area of the infarct attract mesenchymal cells. A larger amount of mesenchymal cells at the damaged tissue after injury can accelerate or modify the

natural injury-repairing processes. It is notable that despite the larger number of living cells in the infarcted tissue and around the necrotic area, the cell proliferation itself was similar in both groups. Therefore proliferation as such seems to be more or less a standard process evoked by initial repair processes and cannot be accelerated by extending the number of BMMCs in the area. Thus, it might be plausible that the number of the cells, per se, is not a defining feature of proliferation; rather, the cells that proliferate might be different.

An interesting finding in our study was that expression of MSA in the labeled cells in the histologic analysis was

detected, which opens the field for the following question: Can these labeled stem cells turn into myocytes? That kind of finding is presented in several other reports.^{5,6} However, the magnitude of the cells expressing MSA is insufficient to explain the recovery of the EF. Thus, as has been suggested,⁹ other mechanisms induced or mediated by the stem cells would be responsible for the healing effect. This idea is also supported by our histopathologic findings showing that the infarcted area in the BMMC group seems to have more muscle actin and mesenchymal cells compared with the infarcted area in the control group. All these data in combination support the possibility that cellular therapy has a locally significant effect on the induction of cell repair.

At 3 weeks after infarction, the healing processes had clearly modified the infarcted myocardium. Local necrosis, the granulation tissue area, and the border zone between the necrotic and granulation tissue areas could be easily identified in the histopathologic analysis. The model used here represents the initial steps of recovery, however, and this should be kept in mind when these data are interpreted: the observed differences between the BMMC group and the control group represent only one time point in a long healing process. Nevertheless, the BMMCs transplanted by means of intramyocardial injection into the infarcted area seem to remain in the heart muscle for the observed 3-week period, and these cells decrease scar formation or modulate scar development. The DiI-labeled and living cells could be found in all named infarct-conjugated areas, but it was obvious that the signs of expression of MSA were mainly concentrated in the granulation tissue area. The labeled BMMCs not only survived at the injection site for 3 weeks but also proliferated, and a fraction of these cells showed positive expression of skeletal muscle actin. The viable infarcted area appeared to be supportive enough for those cells to start the proliferation and other injury-repairing processes. The same processes occurred also in the control group, although to a lower degree. The fact that live labeled cells were detected in the border of the necrotic area and in the necrotic area reveals that the repairing processes initiated by the stem cells cover the area from the outer fringes to the core of the injured area. This was clearly not the case in the control group, which might explain the observed difference between the groups: the center of the damaged area is physically reachable only after a longer period without a direct introduction of cells into the area. It is noticeable, however, that the labeled cells in the severely infarcted area hardly showed any signs of expression of MSA. The quantity of the living cells (Hoechst staining) and Stro-1-positive cells in the necrotic area was significantly higher in the BMMC group, and labeled cells without expression of any specific antibody could be observed in the same area. These data suggest that the unorganized infarcted area is

under such repairing processes that expression of MSA of the labeled cells is neither possible nor induced at this site and also that the viable cells introduced in the damaged tissue might act as signaling centers for the repair process. It is obvious that at least parts of the necrotic areas were beyond repair, and therefore the cellular changes were more pronounced in the viable, sufficiently perfused areas of myocardial tissue.

It has been shown that the smooth muscle actin in the granulation tissue indicates the presence of myofibroblasts and that these cells contribute to the ongoing scar tissue collagen turnover and subsequent fibrous tissue formation.^{19,20} Although the smooth muscle actin staining showed no difference between the groups, in the BMMC group there were perceivably lower amounts of myofibroblasts in the granulation tissue and more in the border zone of the necrotic area. More than 90% of DiI-labeled cells in the organized granulation tissue were α -SMA⁺, whereas in the unorganized granulation tissue only, their proportion was 23%. Hence it appears that the injected cells could have different contributions depending on their location in relation to the damage. It appears that the injected cells were more concretely at work in the organized granulation tissue, and in the unorganized milieu they would exist as signaling centers. As has been described, in the native situation the myofibroblasts proliferate and cover the necrotic area sooner or later.²¹ Hence the injected cells can have an accelerating or facilitating effect on that process. However, it still remains under discussion whether the injected cells can indeed turn the myofibroblasts sooner toward their contractile form.

Our data confirm that transplantation of BMMCs improves the function of the heart and enhances myocardial recovery after severe experimental infarction. The injected cells showed signs of participating in the repair process in all damage-linked areas. The detailed molecular mechanisms that regulate these processes require more profound experimental research, which is still needed to provide optimal therapy for clinical use.

Because of the model used, the clinical relevance of our study is limited. The healing effect in juvenile pigs is probably more pronounced than it would be in adult pigs. The recovery of the function is based on M-mode echocardiography, and it might overestimate the real magnitude of the recovery. It is remarkable, however, that the healing effect was also seen in the histologic analysis and in concordance with the mechanical improvement.

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