Mesenchymal Progenitor Cells Differentiate into an Endothelial Phenotype, Enhance Vascular Density, and Improve Heart Function in a Rat Cellular Cardiomyoplasty Model

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Background—Cellular cardiomyoplasty is a promising approach to improve postinfarcted cardiac function. The differentiation pathways of engrafted mesenchymal progenitor cells (MPCs) and their effects on the left ventricular function in a rat myocardial infarct heart model were analyzed.

Methods and Results—A ligation model of left coronary artery of Lewis rats was used. MPCs were isolated by bone marrow cell adherence. Seven days after ligation, MPCs labeled with 4',6-diamidino-2-phenylindole were injected into the infarcted myocardium (n=8). Culture medium was injected in the infarcted myocardium of control animals (n=529). Thirty days after implantation, immunofluorescence studies revealed some engrafted cells expressing a smooth muscle phenotype (α SM actin), as similarly observed in culture. Other engrafted cells lost their smooth muscle phenotype and acquired an endothelial phenotype (CD31). Furthermore, vessel density was augmented in the MPC group in comparison with the control group. After 30 days, echocardiography showed an improvement on left ventricular performance in the MPCs compared with the control group.

Conclusions—In vivo administration of syngenic MPCs into a rat model of myocardial infarcted heart was safety demonstrated. Some engrafted cells appeared to differentiate into endothelial cells and loss their smooth muscle phenotype. MPC engraftment might to contribute to the improvement on the cardiac function in such a setting. (Circulation. 2003;108[suppl II]:II-253-II-258.)

Key Words: stem cells ▪ myocardial infarction ▪ transplantation

Cellular cardiomyoplasty (CCM) have been the focus of attention over the last several years and become a promising novel therapy for postinfarction impaired heart function (for review, see 1). Over the past decade, a variety of cell types have been proposed such as skeletal myoblasts, 2,3 smooth muscle cells, 4 and mononuclear bone marrow cells. 5 The latter contain among different lineages, hematopoietic 6,7 and mesenchymal stem cells (MSCs), 8,9 which can work beneficially in the ischemic myocardium.

The first clinical trial performing CCM with skeletal myoblasts and mononuclear bone marrow cells seems relevant. 10,11 However, skeletal myoblasts are already committed in the myogenic pathway and bone marrow is not a homogeneous population and containing various cell lineages. Mesenchymal stem cells seem to be highly advantageous for cellular therapy. These particular adherent cells can be expanded ex vivo and, under appropriate culture conditions, have a potential to terminally differentiate into osteocytes, chondrocytes, adipocytes, tenocytes, myotubes, astrocytes, hematopoietic-supporting stroma, and into endothelial cells. 12 The same differentiations have been observed in the experimental models showing the influence of microenvironment for the lineage commitment of implanted cells. 13 Mesenchymal stem cells have been also used in a model of CCM, showing that these cells could differentiate into myogenic cells 9 and enhance angiogenesis.

The aim of this study was to analyze the differentiation pathways of engrafted syngenic mesenchymal progenitor cells (MPCs) obtained in culture from bone marrow MSCs and their effects on the left ventricular function in a rat model of myocardial infarction.

Materials and Methods

Animals

All animals received care in compliance 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 published by the National Institutes of Health (NIH publication 85-23, revised 1996). Both donors and recipients were male Lewis inbred rats weighing 250 to 300 g (Charles River France, l’Arbresle, France).

**Cell Isolation, Culture, and Labeling**

Bone marrow was extruded from tibiae and femurs using a needle. Whole marrow cells were treated with 5 mL of type I collagenase (250U/mL) for 45 minutes at 37°C and washed. Then, cells were cultured at 1.3×10^6/cm^2 in α-MEM supplemented with 10% FBS (Hyclone, lot selected for promoting rapid expansion of MSCs), 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin. The nonadherent cells were removed by a medium change at 48 hours and every 4 days thereafter. The monolayer, referred to as MSCs, was expanded by two passages. Before implantation, MSCs were trypsinized, washed, and labeled with 4’,6-diamidino-2-phenylindole (DAPI, Roche Diagnostic), as previously described. After labeling, DAPI stains specifically 100% of the MSCs nuclei.

**In Vitro Differentiation**

For immunofluorescence studies, 15-day cultured MPCS were treated with trypsin and seeded at 1×10^4 cells/well in plastic laboratory-tek chamber slides (8-well permanox slides, Nunc, Lincolnshire, IL). At confluence, cells were washed once and fixed either with 3.7% (v/v) formaldehyde in PBS or fixed for 30 minutes at 4°C using ice-cold methanol. Both primary antibodies: anti-α SM actin (1A4, Immunotech), anti-vimentin (V9, Sigma), anti-β actin (AC-15, Sigma), anti-CD31 (JC70A, Dako), anti-myosin heavy chain (MHC)(My32, Sigma), anti-desmin (D33, Immunotech), and secondary antibody: antirat polyvalent-PE conjugate (Sigma) were incubated for 30 minutes at room temperature. Irrelevant antibody was used as control. Slides were examined with an Olympus microscope (Viewfinder Lite V1.0, Pixera Co, Berkshire, UK).

**Myocardial Infarction Model and Cell Implantation**

Myocardial infarction was performed by the ligation of the left coronary artery as described previously. Briefly, rats were anesthetized with ketamine (50 mg/kg IP) and xylazine (10 mg/kg IP) and tracheally ventilated with room air using a Colombus ventilator (CTV 101, Colombus Instruments, Columbus, OH). After left lateral thoracotomy in the fifth intercostal space, the left coronary artery was occluded. Ejection fraction was assessed by echocardiography. Then, animals were randomly divided into two experimental groups: control (n=8) and MPCS (n=8).

Seven days after myocardial infarction, cell implantation was performed. Under general anesthesia, a midline sternotomy was performed to expose the heart. The MPCS (1×10^4) DAPI-labeled were suspended in 0.5 mL of medium and were administered by multiple injections into the infarcted area using a 30-gauge tuberculin syringe. The same volume of culture medium alone was injected into the infarcted area in the control rats. Penicillin A (150 000 U/mL) was given intravenously before each operation and buprenorphine hydrochloride (0.05 mg/kg) was administered subcutaneously twice a day for the first 48 hours after operation.

**Functional Assessment by Echocardiography**

Transthoracic echocardiography was performed on animals 6 days after ligation. All rats that have an ejection fraction above 45% were excluded after the first echocardiography examination. Eight rats in each group were included and underwent baseline measurement at this time. Thirty days after cell implantation another echocardiography was performed on 5 rats of each group. Rats were anesthetized with ketamine (50 mg/kg IP) and xylazine (10 mg/kg IP), the chest was shaved, and then placed in the supine position. Echocardiography was performed with a commercially available echocardiographic system (System Five, General Electric) equipped with a 10-MHz linear-array transducer. Any excessive pressure which could induce severe bradycardia was avoided. The echocardiography was performed as described elsewhere. Briefly, the heart was imaged in the 2-dimensional mode in short-axis view of the left ventricle (LV) at the level of the papillary muscle. This view was used to record M-mode tracings. Anterior and posterior end-diastolic and end-systolic wall thickness and LV internal dimensions (LV end-systolic and end-diastolic diameters) were measured according to the American Society of Echocardiography leading-edge method from at least three consecutive cardiac cycles. The LV ejection fraction (LVEF) and the LV percent fractional shortening (FS) were calculated as follows:

\[ \text{LVEF} = \left( \frac{\text{LVDd} - \text{LVDs}}{\text{LVDd}} \right) \times 100 \]

\[ \text{FS} = \left( \frac{\text{LVDd} - \text{LVDs}}{\text{LVDd}} \right) \times 100 \]

All measurements were performed in blind to the treatment group.

**Histological and Immunohistochemical Analysis**

All the rats were sacrificed at day 30 and histological analysis by hematoxylin-eosin, immunohistochemical staining, vascular density analysis, and assessment of the cell grafting were performed. One rat from each group was sacrificed at day 5 and day 20 for histological, immunohistochemical, and vascular density analysis.

Heart sections embedded in paraffin were cut into 5-μm slices and stained with hematoxylin-eosin for histological examination. Serial sections were immunolabeled with antibodies anti-αSM actin, anti-desmin, anti-vimentin, anti-factor VIII (F8/86, Dako), anti-CD31, anti-connexin 43 (Chemicon), and anti-N-cadherin (389, Zymed) followed by incubation with FITC-conjugated antisera.

DAPI positive vessels were counted on 24 sections (2 sections per slide, 2 slides per heart) in the infarct area using a light microscope at ×400 magnification. Five high-power fields in each section were randomly selected and the ratio of number of DAPI-positive vessels of the total number of vessels was calculated, averaged, and expressed as the percentage of DAPI-positive vessels per high-power field (0.2 mm²).

The vascular density was assessed in 12 animals (6 in each group) sacrificed at day 30 after implantation. The number of vessels was counted in blind on 48 sections (2 sections per slide, 2 slides per heart) in the infarct and peri-infarct areas of all animals after staining with an antibody anti-factor VIII using a light microscope at ×400 magnification. Five high-power fields in each section were randomly selected, and the number of vessels in each field was averaged and expressed as the number of vessels per high-power field (0.2 mm²).

**Statistical Analysis**

All values are expressed as mean±SEM. All analysis were performed with appropriate software (Statview, SAS Institute Inc). Comparison of vascular density between groups at day 30 was performed with unpaired Student t test. Differences in cardiac function data were studied using a two-way repeated-measures analysis of variance (ANOVA). If a significant F ratio was obtained, a Bonferroni post hoc test was used to specify pair-wise differences. A value of P<0.05 was considered statistically significant.

**Results**

Myocardial infarction caused approximately 23% mortality within 6 days after coronary ligation occurring mainly during the first 48 hours. We have excluded seven rats after the first echocardiography examination because of incomplete myocardial infarction assessed by a LVEF above 45%. There was no additional death after cell implantation. Because syngenic cell therapy mimics a clinical situation of autologous implantation, animals displayed no evidence for cell rejection.

Anterior septal myocardial infarction was observed in the hearts of all animals. Hematoxylin-eosin staining of cross-sections in light microscopy showed transmural infarction in all animals.
Phenotype of MPCs Before Implantation

Five days after the culture initiation, each MSC formed one colony of MPCs. MPCs appeared morphologically to be a homogeneous population of fibroblastoid cells and maintained similar morphology with passages. Fifteen days after culture, all the cells express vimentin showing their mesenchymal origin. Moreover, they expressed α SM actin and β-actin filaments, which are, respectively, specific of smooth muscle and non-muscle cells, but they did not express CD31, skeletal MHC, and desmin (Figure 1).

Engraftment of MPCs

Analysis of serial paraffin sections did not show any engrafted cells in the noninfarcted myocardium. DAPI-labeled cells were observed 5, 20, and 30 days after implantation in the infarcted area confirming the survival of engrafted cells. DAPI-positive cells were observed in the luminal face of endothelium vessels in all cell implanted hearts. The number of DAPI-positive vessels was approximately 22 ± 3.3% per 0.2 mm². These engrafted cells expressed the endothelial marker CD31 and not α SM actin or desmin (Figure 2).

Vascular Density

In the infarct area, vascular density was significantly higher in the MPCs than in the control group at day 30 postimplantation (8.2 ± 0.4 versus 6.3 ± 0.4, P = 0.001) (Figure 4). Additional experiments performed at day 5 after implantation on one rat of each group, show that this difference can be observed since the fifth day after graft (data not shown). Conversely, in the peri-infarct area, the vascular density at day 30 was not significantly different between the groups (Figure 4).

Left Ventricular Functional Assessment

The LVEF, FS, left ventricle end-systolic diameter (LVESD), left ventricle end-diastolic diameter (LVEDD), and anterior

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Figure 1. In vitro characterization of MPCs after 15 days of culture using immunofluorescence staining with antibodies anti-vimentin and anti-α SM actin (phycoerythrine-conjugated secondary antibody). (A) Before implantation, all MPCs express vimentin showing their mesenchymal origin (x 200). (B) α SM actin positive fine and linear microfilaments were observed in all cells showing smooth muscle cell differentiation pathway (x400).

Figure 2. Evidence for endothelial differentiation of engrafted cells (x 400). Sections have been stained with antibodies anti-CD31 (A and B) and with anti-α SM actin (C and D). Fluorescein-conjugated secondary antibody was used. (A and B) DAPI-positive cells localized in the luminal position of the vessel express CD31 (arrows in B), a marker of endothelial phenotype. (C) Arrow indicates DAPI-positive cell localized in the luminal position of the vessel. (D) DAPI-positive cell does not express α SM actin, perivascular smooth muscle cells are stained by α SM actin.

Figure 3. Evidence for smooth muscle differentiation of engrafted cells (x 200). Sections were stained with antibody anti-α SM actin. Fluorescein-conjugated secondary antibody was used. Thirty days after implantation, DAPI-positive cells expressing α SM actin (head arrow) or not (thick arrow) were observed showing different phenotypes after MPCs implantation. Fine arrows show some vessels stained by α SM actin as positive control.

Figure 4. Vascular density in the infarct area was significantly higher in the MPCs than in the control group at day 30 postimplantation (8.2 ± 0.4 versus 6.3 ± 0.4, P = 0.001). In the peri-infarct area, there was no significant difference between the groups.
wall end-diastolic thickness (AWEDth) measurements at baseline were not significantly different between groups.

Thirty days after implantation, significant improvement on the LVEF and FS was observed in the MPCs compared with control group. There was a significant decrease of LVEF and FS in the control group after 30 days whereas these parameters were steady in the MPCs group (Figure 5). LVESD and LVEDD were significantly smaller in the MPCs than in the control group at day 30. LVESD and LVEDD increased significantly with time in the control but not in the MPCs group (Figure 6). The LV dilatation was decelerated in the MPC group and increased in the control group. The lack of LV dilatation was accompanied by a not significantly improvement on LVEF and FS in the MPCs over time, whereas LVEF and FS were significantly deteriorated in the control group. The reduced AWEDth after coronary ligation was preserved in the MPCs but not in the control group and was significantly higher in the MPCs compared with the control group (Figure 7).

Discussion

The current knowledge of the properties of MSCs has been important for the development of the concept that these cells represent an attractive population for cellular therapy protocols.

The main feature of MSCs is the ability to be isolated from adult bone marrow aspiration and expanded ex vivo before implantation. Bone marrow contains a small number of mesenchymal lineage cells, the MSCs, that grow as adherent myofibroblastic cells in plastic culture.18 These cells can be expanded by culture from many species including rats.19 During their expansion, they acquired a vascular smooth muscle pathway.20 Under differentiation-inducing culture conditions, they have the capacity to differentiate into one or more specialized cells such as bone, cartilage, adipocytes, myocytes (for review see21), and even cardiomyocytes when treated with 5-azacytidine.22

In vivo, authors have shown that MSCs transplanted into myocardium environment, could express myogenic-specific protein such as sarcomeric MHC, desmin, troponin T, and phospholamban.9,15 Tomita et al8 have shown that MSCs can differentiate into myogenic like-cells or be localized in the endothelium. Our results show that some engrafted cells remain SM actin-positive after 30 days, however, these cells did not express connexin 43 nor N-cadherin, two major gap junction proteins of the intercalated disks. This expression has been reported 6 weeks after MSC implantation in a noninfarcted myocardium.15 Further long-term experiments could demonstrate cardiomyocyte terminal differentiation with the formation of intercalated disks within engrafted cells and residual cardiomyocytes.

Other grafted cells were present in the luminal face of endothelium of several vessels and expressed CD31. It has been shown that angiogenesis is an important factor influencing the cell engraftment and the improvement of the ischemic myocardium.23 Kamihata et al15 have shown that bone marrow cells, a mixed population of MSCs, hematopoietic cells, and endothelial progenitors, can in-
duce angiogenesis by supplying angioblasts and angiogenic factors such as VEGF, bFGF, and Ang-1, which contribute to endothelial-lineage cells survival. However, Tomita et al have also shown that the implantation of purified MSCs into the ischemic myocardium enhances angiogenesis. In this study, the authors described the migration of MSCs in the vessel wall, as our study does. Moreover, our results showed that the MPCs was localized in the endothelial luminal position of approximately 22% of vessels and underwent endothelial pathway differentiation, attested by CD31 expression in the DAPI-positive cells. However this result is probably underestimated. The main limitation of using DAPI is the risk of false-negative results of the implanted cells. We have to consider that labeling intensity can be lost with cell division before terminal differentiation. If this were the case, the progeny

Figure 6. Left ventricular diameters after cell implantation. After 30 days, left ventricle end-systolic diameter (LVESD) and left ventricle end-diastolic diameter (LVEDD) were significantly smaller in the MPCs and did not increase over the time compared with the control group. Data are mean±SEM.

Figure 7. Anterior wall end-diastolic thickness (AWEDth) after cell implantation. The reduced AWEDth after coronary ligation was preserved in the MPCs but not in the control group and was significantly higher in the MPCs compared with the control group. Data are mean±SEM.
cells might not be labeled, giving false-negative results that could affect our quantitative results.

Although engaged into smooth muscle cell pathway differentiation by our experimental culture conditions, these cells can lose their α SM actin phenotype when implanted into the ischemic myocardium. These findings confirm the hypothesis that myocardial environment can supply the proper conditions for endothelial differentiation of MSCs. This differentiation may also contribute to the higher vascular density that has been observed at day 30 after implantation in the MPC group. Such vascular density is thought to maintain the viability of the grafted cells and residual cardiomyocytes for successful cellular cardiomyoplasty. The improvement on the LV function by the grafted MPCs was evaluated by echocardiography in our study. Angiogenesis has been shown to contribute to the improvement on myocardial function by the maintenance of the viability of the residual cardiomyocytes and grafted cells. The myocardial function can be also improved by the grafting of smooth muscle cells which can increase or preserve the anterior wall elasticity and thickness after ischemia. Our results show that MPC grafting induced higher vascular density, endothelial and smooth muscle cell differentiation. Taken together, these factors may contribute to the preservation of the anterior wall thickness, the lack of increase in LV dilatation and thus improvement on the LV function.

In conclusion, the efficacy and safety of the implantation of MPCs into a rat model of myocardial infarction was demonstrated. It was shown that engrafted cells acquired endothelial and myofibroblast phenotypes. This differentiation leads to higher vascular density which is associated with the improvement on cardiac function. Cellular cardiomyoplasty with MPCs is clinically relevant in patients with myocardial infarction. Mesenchymal progenitor cells can be isolated easily from adult bone marrow, expanded in vitro, and re-administered to the patient avoiding immunosuppressive therapy. For these reasons, CCM using MPCs might be appear an alternative to myoblasts or embryonic stem cells.

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References

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