Experimental and Clinical Regenerative Capability of Human Bone Marrow Cells After Myocardial Infarction

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Abstract—Bone marrow mononuclear cells (BMCs) from 20 patients with extensive reperfused myocardial infarction (MI) were used to assess their myocardial regenerative capability “in vitro” and their effect on postinfarction left ventricular (LV) remodeling. Human BMCs were labeled, seeded on top of cryoinjured mice heart slices, and cultured. BMCs showed tropism for and ability to graft into the damaged mouse cardiac tissue and, after 1 week, acquired a cardiomyocyte phenotype and expressed cardiac proteins, including connexin43. In the clinical trial, autologous BMCs (78 ± 41 × 10^6 per patient) were intracoronarily transplanted 13.5 ± 5.5 days after MI. There were no adverse effects on microvascular function or myocardial injury. No major cardiac events occurred up to 11 ± 5 months. At 6 months, magnetic resonance showed a decrease in the end-systolic volume, improvement of regional and global LV function, and increased thickness of the infarcted wall, whereas coronary restenosis was only 15%. No changes were found in a nonrandomized contemporary control group. Thus, BMCs are capable of nesting into the damaged myocardium and acquire a cardiac cell phenotype in vitro as well as safely benefiting ventricular remodeling in vivo. Large-scale randomized trials are needed now to assess the clinical efficacy of this treatment. (Circ Res. 2004;95:742-748.)

Key Words: myocardial infarction ■ restenosis ■ myogenesis ■ cardiomyocytes ■ autologous cell transplantation ■ remodeling ■ bone marrow ■ connexin43

Many of the therapies available today, including thrombolysis and urgent revascularization, have significantly improved the prognosis of patients with acute myocardial infarction (MI). However, a high proportion of survivors of this condition are at risk of developing heart failure because of left ventricular (LV) remodeling, a process characterized by mechanical expansion of the infarcted wall followed by progressive LV dilation and dysfunction.

Because the main underlying cause of this process is the irreversible damage of cardiomyocytes and microvasculature of the infarcted wall, the development of treatments aimed at regenerating necrotic myocardial tissue is now considered as a main therapeutic challenge.

Together with recent evidence demonstrating the remarkable ability of adult stem cells to produce differentiated cells from embryologically unrelated tissues, convincing data support that the heart has a potent intrinsic regenerative capacity. In fact, not only cardiac stem cells but also extracardiac progenitors have been shown to regenerate myocytes and vasculature. Consequently, the hypothesis that the natural capability of the mammalian heart to regenerate infarcted myocardium can be reinforced by stimulation of endogenous stem cells, or local delivery of extracardiac progenitors, has led to several investigations suggesting that stem cell therapy could benefit postinfarction LV remodeling. Particularly, bone marrow mononuclear cells (BMCs) homed to infarcted myocardium, induced myogenesis and angiogenesis, and ameliorated cardiac function and survival in animals. Moreover, recent phase I clinical studies and a randomized study showed that intracoronary transplantation of BMCs benefits postinfarction LV outcome in humans. In general, the above observations have been interpreted as attributable to [meta]plastic transformation of the adult stem cells also referred to as transdifferentiation. Alternatively, skeptic views include the proposal of cell fusion as the actual mechanism of tissue repair. In addition, the capability of extracardiac progenitors to transdifferentiate into cardiomyocytes in patients with sex-mismatched infarcted transplanted hearts has been questioned recently and recent studies in mouse showed functional improvement.
of the LV after direct injection of BMCs in the infarcted wall but failed to demonstrate transdifferentiation into cardiomyocytes.\textsuperscript{33,34} Finally, concerns regarding the acute and long-term safety of intracoronary transplantation of BMCs in humans have arisen recently.\textsuperscript{35–39} To shed light on these unresolved controversies, we have undertaken an investigation in which BMCs from patients with acute MI were used for a clinical trial and for a parallel study of grafting and differentiation in vitro. The in vitro study evaluated the ability of the human BMCs to home and graft into cryoinjured mouse heart slices and to acquire a cardiac cell phenotype. The clinical trial was designed as a phase I safety and feasibility study for testing the effects of intracoronary administration of autologous mononuclear BMCs on the integrity of the coronary tree and postinfarction cardiac performance.

Materials and Methods

Patients and Protocol

The ethics committee of our hospital approved the study protocol. Patients were consecutive and prospectively included between September 2002 and November 2003 after giving written informed consent. Inclusion criteria were: (1) age between 18 and 75 years; (2) extensive ST-elevated MI (baseline total ST elevation \textsuperscript{6}mm) treated with or without rescue thrombolysis; (3) successful reperfusion with 2% autologous plasma in Teflon bags (Life-cell; Baxter). The BMC suspension was infused with a pump at 1 to 2 mL per minute during periods of 3 minutes of inflation and cell infusion alternating with 1 minute of deinflation and reperfusion until the total dose of BMCs (10 to 25 mL) was given.

Preparation of Progenitor Cells

Bone marrow (\textasciitilde50 mL) was aspirated under local anesthesia from ilium. BMCs were isolated by Ficoll density separation and cultured overnight at 1x10^6 cells/mL in RPMI-1640 culture medium (Gibco) with 2% autologous plasma in Teflon bags (Life-cell; Baxter). The next day, BMCs were harvested, washed, and resuspended at \textasciitilde5x10^6 cells/mL in heparinized saline. BMCs were immunophenotyped by flow cytometry (FACScalibur; Becton Dickinson Biosciences) for CD34, CD117, and CD-133 just before infusion. About 95% of the BMCs were used for intracoronary infusion and 5% for the in vitro experiments.

Preparation and Culture of Mouse Heart Slices

Male mice (BALB/c, 12 weeks old) were killed by cervical dislocation and decapitation. After exsanguination, the heart was perfused with 4% agar in PBS at 37°C. The tissue was cooled to 10°C, atria were removed, and 250-μm-thick sagittal slices of the ventricle were prepared using a Peclod Mod. 1000+ vibratome. After removing agar by washing with warm PBS, a cryosection was produced near the apex by touching with a 1-mm-diameter metal bar cooled in liquid nitrogen. Slices were placed on top of Nunc 25-mm tissue insert (category No. 136897) and cultured in RPMI 1640 medium containing 10% FBS and antibiotics at 37°C under water-saturated 5% CO2/95% air atmosphere. After 12 to 24 hours in culture, human BMCs were added on top of the slice and cultured for up to 3 weeks. The experiment was terminated by fixing the slices with 4% p-formaldehyde/0.2% picric acid in PBS for 1 to 3 hours. Then, 5-μm cryosections were prepared and processed for immunohistochemistry as described below.

Fluorescent Staining and Immunohistochemistry

BMCs were stained with 20 μmol/L cell tracker (chloromethylene-benzamido dioctadecyl-tetramethylindolocarbocyanine; Molecular Probes) in culture medium containing serum for 30 minutes at 37°C. Cryosections were stained with either monoclonal anti-α-sarcromeric actin (clone 5C5; Sigma), monoclonal anti-α-actinin (sarcomeric; Sigma), rabbit polyclonal anti-connexin43 (H-150; Santa Cruz Bio-technology), or anti-α-smooth muscle actin (Sigma). The appropriate secondary antibodies conjugated with Alexa 488, fluorescein isothiocyanate (FITC), or rhodamine were used, coverslips were mounted with Vectashield H-1000 (Vector Labs), and cells were examined with the appropriate filters for immunofluorescence. Nuclei were stained with 4’,6-diamidino-2-phenylindole.

Intracoronary Transplantation of BMCs

An over-the-wire balloon catheter positioned at the site where the stent had been implanted was inflated at 2 to 4 atmospheres until complete block of blood flow. Then, the guide wire was retired, and the BMC suspension was infused with a pump at 1 to 2 mL per minute during periods of 3 minutes of inflation and cell infusion alternating with 1 minute of deinflation and reperfusion until the total dose of BMCs (10 to 25 mL) was given.

Magnetic Resonance

Patients were imaged by means of a General Electric Signa 4.0 scanner operating at 1.5 T with a body coil and ECG triggering. Image acquisition protocol was as described previously,\textsuperscript{40} and LV ventricular volumes and ejection fraction were calculated and blindly analyzed with MASS software. A 16-segment model was used, and each ventricular segment was given a score according to its motion as proposed.\textsuperscript{41} Regional function was assessed by assigning a score to each segment according to its motion: I normal, 2 hypokinetic, 3 akinetic, and 4 dyskinetic. Wall motion score index was calculated from the sum of the segmental scores divided by the segments visualized. Intraobserver variability in our laboratory was as follows: LV end-diastolic volume 9.5±10.5 mL (0.06±0.05%), LV end-systolic volume 7.6±8.2 mL (0.10±0.09%), and ejection fraction 4.1±3.6 (0.08±0.07%). Interobserver variability was 11.9±13.5 mL (0.07±0.08%), 9.2±9.5 (0.12±0.12%), and 2.9±2.1 (0.06±0.05%), respectively. Agreement in regional assessment of contractility was 95%. End-diastolic and end-systolic thickness was calculated at the thinnest point in the area with asynergy. Thickening was calculated by the formula end-systolic thickness minus end-diastolic thickness.

Low-Dose Dobutamine Stress Echocardiography

Dobutamine was given intravenously at an initial dose of 5 μg/kg per minute that was incremented every 3 minutes in 5 μg/kg per minute until a maximal dose of 20 μg/kg per minute was completed. Continuous ECG was recorded and semiquantitative regional function was blindly analyzed as aforementioned. Agreement in regional assessment of contractility was 93%.

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Cardiac Catheterization and Angiographic Assessment
CFR thermo and FFR were assessed as described previously. Quantitative angiographic computer-assisted analysis (QCA-CMS/3.0 and QLVA/4.0 from MEDIS-Medical Imaging System) was performed at an independent core laboratory by experts who were unaware of patients’ clinical outcome. Complex coronary lesions and thrombus were defined as usual. A “de novo” or restenotic lesion was considered significant if it provoked a reduction ≥50% in the normal reference diameter.

Statistical Analysis
Except when otherwise stated, data are presented as mean±SD. Statistical significance was set at P<0.05. A P value between 0.05 and 0.1 was considered to approach significance. Intraindividual comparison of baseline versus follow-up continuous variables was performed with a paired t test. Cell therapy versus control group comparisons were compared by Student t test, and Fisher exact test when necessary. Adjustment to a normal distribution was checked previously by the Saphiro-Wilk test.

Results
Table 1 compares the baseline characteristics of the 20 transplanted patients and the 13 controls. All patients underwent stenting within 24 hours of thrombolysis (tenecteplase), performed with similar delay in transplanted patients and controls (175.4±103.7 versus 196.9±105.4 minutes after infarct onset, respectively; P=0.69). No untoward events occurred between reperfusion therapy and BMC transplantation. An average of 78±106 BMCs were transplanted per patient (range 11 to 175×10⁶) into the culprit artery 13.5±5.5 days after the infarction (range 5 to 29 days). The phenotypic characteristics of the injected cells were as follows: CD34⁺, 1.0±0.6%; CD117⁺, 1.7±0.9%; and CD133⁺, 0.6±0.3%.

Human BMCs Integrate Into Damaged Myocardium and Acquire Myocyte Characteristics In Vitro
To test the cardiogenic potential of human BMCs, a sample of the same cell preparation used for transplantation from patients in the treated group containing ~2×10⁶ cells was labeled with CM-DiI cell tracker, seeded on top of cryolesioned mouse heart slices, and cultured for up to 3 weeks (Figure 1A). The DiI-labeled BMCs could be traced by their red fluorescence along the whole experimental period (Figure 1B). In the first few days, the DiI-labeled cells looked rounded and uniform and located on top of the cardiac slice with a detectable concentration over the cryolesion. After 1 to 2 weeks of coculture, a subpopulation of the BMCs enlarged and looked progressively more complex (Figure 1C). These cells remained preferentially near the lesion area, where they integrated into myocardium (Figure 1D). A significant fraction of BMC-derived cells expressed cardiac-specific proteins, such as connexin43 (Figure 1E) or sarcomeric actin (Figure 1F). No evidence of expression of proteins specific for smooth muscle (smooth muscle actin) was found (data not shown). More than 80% of the surviving BMC-derived cells, as assessed by DiI labeling after 1 week, were located in the vicinity of the damaged myocardium.

Safety Data and Clinical Follow-Up
Mean follow-up after transplantation was 11±5 months (range 6 to 21 months). No patient experienced periprocedural complications or increase in myocardial injury markers at 24 hours (Troponin T before and 24 hours after cell therapy: 0.23±0.48 ng/mL and 0.22±0.47 ng/mL, respectively; P=0.25; CK-MB before and after cell therapy 15.8±3.0 and 15.0±4.0, respectively; P=0.46). Intracoronary cell infusion induced no changes in the FFR (0.91±0.06 and 0.88±0.05 before and after cell infusion, respectively; P=0.34) and the CFR thermo (2.17±0.87 and 2.02±0.43 before and after cell infusion, respectively; P=0.70). Neither major cardiac events nor spontaneous or stress-induced arrhythmias were observed during the follow-up. Moreover, all cases remained free of major cardiac symptoms or events. Two patients required stenting at 5 months because of progression of previous nonsignificant stenosis in a noninfarct-related artery. Another patient experienced a transient stroke 1 month after the procedure without sequelae.

LV Outcome
As depicted in Table 2, 6-month MRI showed no increase in the end-diastolic LV volume. Moreover, a significant im-
**LV Contractile Reserve**

The end-diastolic and end-systolic thickness of the infarcted wall measured by MRI increased significantly at 6 months (Figure 2). Similarly, infarcted wall thickening rose from $2.0\pm 1.0$ mm up to $3.2\pm 1.5$ mm. Concordantly, dobutamine-induced increment in ejection fraction tended to be significantly larger after 6 months than in the baseline study ($9.61 \pm 4.78$ vs $2.0 \pm 1.5$ mm).Parallel in vitro experiments with the same population of human BMCs used for the clinical trial clearly show that these cells are able to graft into injured mouse myocardium. BMCs in close proximity to the damaged mouse myocardium might be able to regenerate contractile myocardial tissue. The capability of a subpopulation of BMCs to differentiate into cardiomyocytes is a crucial but controversial biological question. Several experimental reports have documented the formation of new myocytes and vascular cells from genetically marked cells injected into damaged myocardium and clinical studies, particularly of heart and bone marrow transplants, have also supported this concept. In contrast, others have been unable to obtain myocyte differentiation in the mouse model and, therefore, deny the possibility of BMCs to regenerate myocardial tissue. In the clinical research side, 7 phase I clinical trials and a

**Coronary Angiography**

Baseline and poststenting angiographic characteristics of transplanted and control patients are depicted in Table 1. At 6 months, poststenting late loss in actively treated patients was $0.67 \pm 0.34$ mm, and significant restenosis was observed in 2 patients (10%). Besides, in 2 patients, an initially nonsignificant stenosis located in a nonculprit artery became severe during follow-up (10%). In the control group, 6-month angiographic follow-up revealed a late loss of $0.66 \pm 0.78$ mm ($P=NS$ versus the transplanted group), and 2 patients developed binary significant restenosis (15%; $P=NS$ versus the transplanted group).

**Discussion**

Our results show that intracoronary transplantation of autologous BMCs after acute MI is feasible and safe in the short and midterm. In addition, this intervention seems to significantly diminish the extent of LV remodeling and promote a significant recovery of cardiac function. Although the mechanism(s) of this putative beneficial effect remain to be elucidated, our data suggest that BMC therapy for acute MI might be able to regenerate contractile myocardial tissue. The end-diastolic and end-systolic thickness of the infarcted wall measured by MRI increased significantly at 6 months (Figure 2). Similarly, infarcted wall thickening rose from $2.0\pm 1.0$ mm up to $3.2\pm 1.5$ mm. Concordantly, dobutamine-induced increment in ejection fraction tended to be significantly larger after 6 months than in the baseline study ($9.61 \pm 4.78$ vs $2.0 \pm 1.5$ mm).
randomized trial\textsuperscript{22} using autologous BMCs have uniformly shown a preliminary beneficial effect of this therapy.

Three available lines of evidence converge in favor of the interpretation that the transplanted BMCs participate directly and indirectly in the regeneration of cardiac myocytes and microvasculature post-MI: (1) the recent observations that cardiac endogenous stem cells present in the normal myocardium and involved in the maintenance of the cardiac cellular homeostasis are also able to expand and regenerate myocytes and microvasculature in the infarcted myocardium;\textsuperscript{5} (2) the evidence that cardiomyocyte repopulation by extracardiac progenitors of hematopoietic origin can take place in the human;\textsuperscript{9,44–46} and (3) the demonstration that it is possible to increase the efficiency of the intrinsic cardiac regeneration capacity in animals with acute MI by both local delivery of BMCs\textsuperscript{11,14} and bone marrow mobilization with cytokines,\textsuperscript{12} resulting in a reduction of infarct size and clear improvement in LV performance and survival. From the above, it is reasonable to argue that the transplanted BMCs are likely to stimulate the endogenous regenerative myocardial processes, perhaps through a combination of a paracrine effect that stimulates the expansion, homing, and differentiation of endogenous cardiac stem cells\textsuperscript{47} together with differentiation toward cardiac phenotype and regeneration of necrotic myocytes and vascular cells. Our finding that the same BMCs given to the patient are able to graft into the mouse myocardium slices and acquire the phenotype of parenchymal heart cells are consistent with this view. This simple in vitro model allows a detailed follow-up of the fate of BMCs and circumvents the immunological problems derived from testing the human cells in intact animals. We have shown that human BMCs integrated effectively into surrounding mouse myocardial tissue and, after 1 week, acquired the phenotype of parenchymal heart cells are consistent with this view. This simple in vitro model allows a detailed follow-up of the fate of BMCs and circumvents the immunological problems derived from testing the human cells in intact animals. We have shown that human BMCs integrated effectively into surrounding mouse myocardial tissue and, after 1 week, acquired the phenotype of parenchymal heart cells. Our finding that the same BMCs given to the patient are able to graft into the mouse myocardium slices and acquire the phenotype of parenchymal heart cells are consistent with this view. This simple in vitro model allows a detailed follow-up of the fate of BMCs and circumvents the immunological problems derived from testing the human cells in intact animals. We have shown that human BMCs integrated effectively into surrounding mouse myocardial tissue and, after 1 week, acquired the phenotype of parenchymal heart cells. This is so because fusion of transplanted cells to resident myocytes does not generate new myocytes as the fused cell is unable to re-enter the cell cycle. Our results do not give additional information on this heat controversy. However, despite the evidence that cell fusion occurs both in vivo and in vitro,\textsuperscript{23–31} its frequency has been suggested to be lower than would be required to have a significant regenerative effect.\textsuperscript{49}

The clinical results are consistent with the in vitro observations. As in similar studies,\textsuperscript{15,16,22} we found that autologous BMCs intracoronary transplantation after MI significantly reduces LV remodeling and improves both global and segmental cardiac performance. We decided to administer the BMCs later than in other studies (13.5 ± 5.5 versus 4 to 7 days postinfarction) to prevent their transformation into inflammatory elements.\textsuperscript{50} Importantly, MRI showed an increase in the thickness and the thickening of the infarcted wall. Because baseline low-dose dobutamine stress echocardiography was negative for viability, it seems unlikely that the thickening may be attributable to compensatory hypertrophy or improvement in stunned myocardium.\textsuperscript{51} Moreover, the observed increase in thickness, a finding not reported so far, strongly suggests that myocardial regeneration occurs after cell transplantation, given that recovery of stunned myocardium should not be associated to such structural change.

Concerns regarding short- and long-term safety of BMC-based cell therapy question the appropriateness of conducting clinical randomized trials aimed at assessing the efficacy of such treatment in patients with recent MI.\textsuperscript{16,37} First, bearing in mind the deleterious effect of persistent microvascular dysfunction on postinfarction LV remodeling and clinical outcome,\textsuperscript{3} the potential effect of BMC intracoronary infusion on producing or aggravating no reflow by microvascular embolic occlusion requires cautious analysis. After intracoronary infusion of mesenchymal cells in noninfarcted dogs, Vulliet et al\textsuperscript{35} observed ischemic changes, myocardial injury, and microinfarctions with accumulation of the infused cells. Likewise, Kang et al\textsuperscript{37} recently reported a mild increase in myocardial injury markers after intracoronary transplantation of BMCs. In contrast, like in other studies,\textsuperscript{15,16} we observed neither myocardial injury nor microvascular dysfunction after
transient repetitive occlusion and simultaneous slow infusion of mononuclear BMCs into coronary arteries supplying non-viable injured areas. Second, although circulating hematopoietic progenitors contribute to the integrity of endothelium and benefit re-endothelialization after arterial damage, data have been reported suggesting that BMC therapy could induce progression of de novo and restenotic coronary lesions. Particularly, Kang et al. reported recently an unexpectedly high rate of in-stent restenosis in patients with recent MI who received G-CSF for bone marrow mobilization alone or associated with intracoronary infusion of BMCs. By contrast, in our study, intracoronary infusion of BMCs produced neither more restenosis nor more atherosclerosis progression than expected. Even if cell transplantation is regarded as responsible for the development of the 2 restenotic and 2 nonrestenotic significant lesions seen at 6 months, 20% of significant stenosis 6 months after stenting in the setting of reperfused acute MI is acceptable and lower than that reported in the literature. Therefore, altogether our results support previous evidence suggesting a favorable risk-benefit ratio for intracoronary administration of autologous BMCs that allows development of large-scale randomized clinical trials in this area.

Several limitations of this study have to be addressed. It should be stressed first that this study was designed as a phase I safety and feasibility trial; it did not include a randomized control group. To validate the results, we correlated them with those obtained from a contemporary similar group of patients who received standard therapy. Although such a comparison does not reach the power of a randomly allocated, blinded control group, our results suggest that the improvement observed is attributable to the treatment. Secondly, dobutamine stress echocardiography is not the ideal technique to detect viability. Sensitivity varies from 70% to 85% and specificity from 80% to 90%. However, these values were reported before second harmonic imaging was available, which was shown to improve substantially the accuracy of this technique. Besides, when compared with the remaining imaging techniques, including positron emission tomography and scintigraphy, dobutamine stress echocardiography has the highest positive and negative predictive values. Thus, in the absence of direct information from necropsy, dobutamine stress-echocardiography remains as a valuable tool to assess viability.

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