Transplantation of Autologous Fresh Bone Marrow Into Infarcted Myocardium: A Word of Caution

Alain Bel, MD; Emmanuel Messas, MD; Onnik Agbulut, PhD; Patrice Richard, MD; Jane-lyse Samuel, PhD; Patrick Bruneval, MD; Albert A. Hagège, MD, PhD; Philippe Menasché, MD, PhD

Background—As the benefits of extemporaneous transplantation (Tx) of fresh (unfractionated) autologous bone marrow (BM) have been primarily studied in the setting of acute myocardial infarction, we assessed whether this approach could be effective for regenerating chronically infarcted myocardium.

Methods and Results—Myocardial infarction was created in 18 sheep by ligation of circumflex arterial branches. Three weeks later, BM was aspirated from the iliac crest, washed, labeled with the fluorescent dye Dil and reinjected (mean: $422 \times 10^6$ cells in 3 mL) in 10 sites across the infarcted area through the reopened thoracotomy ($n=9$). Nine controls received culture medium. Left ventricular (LV) function was assessed before and 2 months after Tx by two-dimensional echocardiography whereas transmural velocity gradients were measured using M-mode tissue Doppler imaging at the center of the infarcted/grafted area. Formalin-fixed hearts were processed for the detection of grafted cells and angiogenesis. LV ejection fraction deteriorated similarly in the Tx and control groups (from 42±5% to 30±4% and from 40±4% to 31±1%, respectively; $P=0.86$). Likewise, BM Tx failed to prevent LV dilatation and impairment of the global wall motion score. The decrease in regional systolic velocity gradients ($s^1$) featured a similar pattern (Tx group: from 0.77±0.11 to 0.31±0.07; control group: from 0.73±0.10 to 0.50±0.07; $P=0.06$). Histologically, there was neither BM tissue engraftment, except for a few scattered Dil-positive macrophages in the infarcted fibrotic areas nor transdifferentiation of BM cells into endothelial cells.

Conclusion—These data caution against the functional efficacy of extemporaneous Tx of fresh unfractionated BM into postinfarction scars. (Circulation. 2003;108[suppl II]:II-247-II-252.)

Key Words: transplantation ■ stem cells ■ bone marrow ■ cell therapy ■ myocardial infarction

Cellular transplantation has recently emerged as a potential new treatment of ischemic cardiomyopathy. Experimental studies have demonstrated improvement in global left ventricular (LV) function following engraftment of cultured adult skeletal muscle precursor cells (myoblasts) in infarcted myocardium. However, a potential limitation to the efficacy of myoblasts is their maintained commitment to a skeletal muscle-type lineage without any transdifferentiation into cardiac or endothelial cells. In contrast, bone marrow (BM)-derived stem cells are currently raising a great deal of enthusiasm because of a purported plasticity which would allow them to change their phenotype in response to cues provided by the target organ. From a practical standpoint, the use of BM is also attractive because of the possibility of using the patient’s own cells and, on the basis of encouraging animal data, some clinical studies have been recently published which advocate the simple extemporaneous reinjection of unfractionated BM in patients with acute myocardial infarction or critical lower limb ischemia. However, as these experiments have been performed at the acute stage of the ischemic insult, their relevance to chronically infarcted myocardium remains uncertain. The present study was therefore designed to address this issue in a sheep model of myocardial infarction undergoing transplantation of autologous fresh (unfractionated) BM. Both global and regional function were assessed using echocardiography and tissue Doppler imaging (TDI), respectively, and these changes were correlated with histopathological data.

Materials and Methods

All experiments were performed in accordance with the “Guiding Principles in the Care and Use of Animals” approved by the American Physiological Society.
Myocardial Infarction

Male sheep (45 to 50 kg) were supplied by the Institut National de la Recherche Agronomique (Paris, France). Under anesthesia (thiopental sodium, 15 mg/kg) and tracheal ventilation, a left thoracotomy was performed in the fourth intercostal space and myocardial infarction was created by ligation of all obtuse marginal branches of the circumflex artery.

Cell Transplantation

Fresh BM was obtained by iliac crest aspiration before completion of the baseline assessment. Bone marrow was aspirated (100 mL) from approximately 10 sites using heparinized syringes (20U heparin/1 mL fresh BM) and was immediately diluted with an equal volume of RPMI medium (Life Technologies, Cergy Pontoise, France). The red blood cells were removed by density-gradient centrifugation after adding an equal volume of Ficoll solution (Eurobio, France) to the diluted BM samples and centrifuging at 1200g for 20 minutes. Following centrifugation, the low-density fraction was collected and rinsed with RPMI medium. Cells were incubated first at 37°C for 5 minutes and then for 15 minutes with the fluorescent probe 1,1-dioctadecyl-3,3,3,3-tetramethyl indocarbocyanine perchlorate (Dil-Ac-LDL, Molecular Probes, Eugene, CA) at a final concentration of 1 μg/mL. The cells were then rinsed at least two times in RPMI medium to remove all excess of fluorescent dye before transplantation. Bone marrow cells were resuspended in 3 mL medium and kept on ice until they were implanted, usually within 10 minutes of collection.

Intramyocardial Injections

Following infarction, sheep were randomized to receive culture medium only (n=9, control group) or cells (n=9, BM group). Three weeks after infarction, the left thoracotomy was re-opened under general anesthesia. Irrespective of the cell number, ten intramyocardial injections of 0.3 mL each were performed across the infarcted area (delimited by superficial 8/0 polypropylene stitches) using a 27-gauge needle.

Assessment of Results

Characterization of the Bone Marrow Injectate

The percentage of mononuclear cells was determined by counting after May–Giemsa staining. An aliquot of the injectate was then cultured on Dulbecco’s modified Eagle’s medium (DMEM) supplemented with fetal bovine serum (15%) and mesenchymatous cells were identified through their adhesive properties. A more accurate phenotypic characterization of the mixed cell yield was not possible because of the lack of sheep-specific antibodies directed against surface markers.

Ultrasonic Assessment

Acquisitions

Under general anesthesia, epicardial echocardiography was performed using a 5.0 MHz probe (5V2) for harmonic imaging or a 3.5 MHz probe (3V2c) for TDI, connected to a Sequoia 512 system (Acuson, Mountain View, CA). Examinations were performed (1) at the time of infarction; (2) immediately before injections, three weeks (W3) after infarction; and (3) 2 months after injections (M2). One parasternal LV long-axis view (including the mitral and aortic valves and the apex) and three parasternal short-axis views (base, papillary muscles and apex) were recorded. For TDI studies, the mid-ventricle parasternal short-axis view was optimized to display the posterolateral wall, constantly involved by the infarction, just below the posterior mitral papillary muscle. Color-coded tissue velocity data were superimposed onto conventional two-dimensional images. The gray scale was diminished and the zoom feature was used to enlarge the visualization of this segment. M-mode TDI images were obtained at different Nyquist velocities at 200 mm/sec with the cursor perpendicular to this segment. Tissue velocity values were fixed just below the Nyquist limits and the Doppler color gain was adjusted in order to obtain an optimal filling of the myocardium, without saturation or aliasing.

LV Volume and Ejection Fraction Calculations

Systolic and diastolic LV long axis (L) were measured on the long-axis view and systolic and diastolic short-axis LV endocardial areas traced at the mitral (A1), papillary muscles (A2) and apex (A3) levels. Left ventricular end-diastolic (LVEDV) and end-systolic (LVESt) volumes were calculated using the simplified Simpson’s rule as (A1+A2)/2h + (A3/2)h + mL/6 with h=L/3 and LV ejection fractions (EF) were then calculated.

Scoring of Regional Contractility

Using the three short-axis views, the LV was divided into 16 segments and a score was allocated to each segment according to its contractility as (0) normokinetic, (+1) hypokinetic, (+2) akinetic, (+3) dyskinetic. A global wall motion score (WMS) was derived from the algebraic sum of these values.

TDI Studies

In each stored image, the posterior wall was divided into two layers of equal thickness by manual tracing of endocardial and epicardial boundaries. The computer program (Datapro®, Acuson, France) converted color-encoded myocardial velocities into velocity estimates and the myocardial velocity gradient (MVG) (s⁻¹) was then calculated along the M-mode line as the difference between endocardial and epicardial velocities divided by wall thickness, as previously described. The peak values of the MVG were determined in systole during early LV ejection and in early diastole during rapid LV filling. All measurements were a mean of three consecutive beats by two experienced operators who were blinded to the treatment group.

Pathologic Assessment

Two months after transplantation and after the last echocardiographic assessment, the animals were sacrificed and hearts were removed. Each transplanted area was easily identified as a transmural infarction. The whole infarcted tissue was fixed in 10% formaldehyde for 48 hours and embedded in paraffin for histology. Five-μm-thick sections were stained with hematoxylin-eosin for qualitative histological analysis specifically targeted at assessing the presence of scar tissue, inflammation and in-scar donor-derived “new” cardiomyocytes. Perls staining was used to identify intracellular iron overload in mononuclear cells present in scar tissue and served as an indirect marker for sheep macrophages as the unavailability of antibodies specific for this species precluded more specific immunophenotyping.

To detect the labeled grafted cells, the unstained sections were observed using a fluorescence microscope (Leica, Rueil-Malmaison, France) with Texas red filter (range of the wavelength: 595 to 615 nm).

Statistics

All data are reported as mean ± SEM. Statistical analysis used StatView 5.0. Groups were compared before injections by a Student unpaired t test, checking for similarity of baseline data. Comparison of variables between baseline, W3, and M2 used the Student paired t test. Groups were compared at M2 using the Student unpaired t test. A probability value <0.05 was considered as significant.

Results

Myocardial infarction was created in 23 animals. Five animals were excluded because of lethal ventricular fibrillation at the time of coronary ligation, which left a total group of 18 animals (9 controls and 9 transplanted with BM) available for serial functional studies until sacrifice.

Bone Marrow Cells

The average number of injected cells was 422±103 million (range, 90 to 900) with a ≥90% viability, as assessed by the blue trypan dye assay. This unpurified bone marrow cell mix
Two months after unfractionated BM cell transplantation, global LV function of similar magnitude (Figure 2). In keeping with these global function data, the TDI-based assessment of regional function showed a similar impairment in the wall motion score (transplant group: from 12.4±1.6 to 16.1±1.9; control group: from 11.6±1.1 to 15.3±1.3; P=0.43) and in the myocardial velocity gradient (transplant group: from 0.778±0.115 to 0.311±0.075; control group: from 0.738±0.105 to 0.500±0.078; P=0.06). Likewise, the extent of remodeling, reflected by an increase in LVEDVs, was unaffected by BM cell injections (transplant group: from 43.1±3.3 to 57.0±6.7; control group: from 44.8±2.6 to 52.7±4.4; P=0.38) (Table 1).

**Discussion**

The major finding of this study is that transplantation of fresh unfractionated BM into postinfarction scars fails to induce any differentiation of grafted cells into cardiomyocytes or endothelial cells, which correlates with a lack of functional benefit compared with control injections of culture medium alone.

Because assessment of regional function is critical in proof-of-concept cell therapy experiments, the present study largely relied on TDI data. Namely, although conventional ultrasound technique is widely used for the evaluation of regional LV function as it depicts in real time wall motion (endocardial excursion and myocardial thickening), it is only a qualitative method that is both subjective and observer-dependent. In contrast, TDI, which directly measures in-plane components of myocardial deformation, has been validated for the computerized quantification of regional myocardial function and has proven to be highly sensitive and specific for the detection of decreased myocardial function due to ischemic or cardiomyopathy processes. Thus, the results of our blinded analysis showing the absence of between-group difference in posttransplantation TDI data provide compelling evidence that BM cell injections failed to preserve postinfarction function. This conclusion is consistent with the histological data showing similarly fibrotic areas in the two groups, without evidence for a new.
in-scar cardiac tissue that might have derived from the grafted cells. The presence of scattered in-scar fluorescent macrophages is most likely inconsequential as although these cells might be remnants of the initial injectate or native inflammatory cells that had re-uptaken the fluorescent dye released by injected dead cells, the fact that a similar pattern was found in control hearts which were not injected with the dye rather suggests some cellular autofluorescence. In any case, these fluorescent cells are not expected to be functionally effective. Overall, these observations dramatically differ from our previous findings that skeletal myoblasts injected into postsin-frac- tion scarred areas differentiate into myotubes which tend to replace fibrosis and that this engraftment correlates with a significant improvement of LV function, both at the regional and global level.4

Previous studies, however, have reported more positive data following injections of BM-derived mononuclear cells. The discrepancy between these results and ours should be analyzed in light of major differences in protocol design, as this may help in defining the place of BM transplantation in the setting of myocardial infarction. Thus, Hamano et al,8 using a porcine model of permanent coronary artery ligation, also failed to show new angiogenesis or improved wall motion in the infarcted area implanted with autologous unfractionated BM cells and reported that these beneficial effects were confined to the marginal area intermediate between infarcted and normal myocardium. These effects, however, were not of sufficient magnitude to translate into an improvement of global hemodynamics. In contrast, Kamihata et al9 has reported an increase in ejection fraction following injection of mononuclear cells very shortly (60 minutes) after coronary artery ligation in swine. Put together, these data suggest that to be functionally effective, extemporaneous injection of BM-derived unfractionated cells should possibly be performed at the early stage of infarction, ie, at a time where there is still living peri-infarct tissue that may harbor the appropriate signals for driving the purportedly pluripotent component of the grafted cells toward an endothelial and/or cardiomyogenic differentiation pathway. Transplant-derived benefits could then derive from angiogenesis and subsequent salvage of reversibly damaged host cardiomyocytes or generation of new cardiac cells, respectively. However, these differentiation cues are likely to be lost at the late stage of chronic scar which is then made of a fibrous extracellular matrix and assuming that implanted BM cells are sensitive to environmental signals, the risk is even that they convert to fibroblasts, which has actually been observed.17,18 This concept of an early injection of unpurified BM cells has been endorsed by the clinical investigators10,11 who have reported preliminary encouraging outcomes following intracoronary administration of these cells in patients with acute myocardial infarction, although the efficacy of this approach must be weighed against the potential for an increased inflammatory cell death rate if injections are too close to the ischemic event.19 Indeed, it is likely that there is an optimal time window that needs to be fine tuned. The coincident assumption that the efficacy of BM transplantation requires target tissues to be reversibly damaged, as opposed to irreversibly necrotic, is also supported by the improvement in coronary perfusion following intramyocardial surgical20 or endocardi-al21 injections of unfractionated BM in patients with ischemic symptoms. These data are paralleled by the increase in neovascularization induced by intramuscular injections of fresh mononuclear cells in rats22 and patients12 with severe lower limb ischemia.

However, apart from the timing (early versus late) and precise site (border zone versus core of the infarction) of BM transplantation, the nature of the cell yield needs also to be discussed. In this study, we used a total unpurified mononuclear cell yield because this is a clinically appealing procedure which is simple to implement and avoids the risks, time and costs related to culture and expansion techniques. This cell population is indeed a complex mix which contains both CD34+, CD34−, endothelial progenitors and stromal cells and although it has been postulated that co-administration of these various subpopulations could potentiate their respective advantages,12 our negative results could alternatively be explained by the small percentage of pluripotent cells “diluted” in this heterogeneous injectate. Theoretically, this issue can

Abbreviations

EDV indicates end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction; WM score, wall motion score; MV gradient, myocardial velocity gradient.

Data for Bone Marrow Group, Pretransplantation and Posttransplantation

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be addressed by selecting subpopulations of hematopoietic or stromal stem cells.

The first approach is based on the assumption that the greater the immaturity of the bone marrow cells, the greater their pluripotentiality, which would justify to sort cell subpopulations located at the “upstream” level of the differentiation scale to enhance the likelihood that environmental factors will then switch their phenotype toward that of the host tissue. In support of this hypothesis, administration of c-kit−/Lin− or CD34+ progenitors by direct intramyocardial implantation,23 intravenous injections,24 or cytokine-induced endogenous mobilization25 has been reported to “regenerate” infarcted myocardium through formation of new cardiac, smooth and endothelial cells23 as well as limitation of apoptosis,24 with the caveat that cell therapy was again implemented within a few hours (9 to 48 hours) following onset of infarction. Regardless of the timing of their administration, another limitation of progenitors is their small number in the circulating blood (1% to 2% for the whole CD34+ population), which probably accounts for the data of Jackson et al.,26 reporting an engraftment rate in periinfarct border zones of only 0.02% and 3% for donor-derived cardiomyocytes and endothelial cells, respectively. Scale-up is thus mandatory to optimize the functional efficacy of this approach but is not trivial to achieve as in vivo mobilization by cytokine treatments may not be innocuous in patients with acute myocardial infarction, whereas in vitro expansion procedures may compromise the differentiation potential of progenitors.27

On the other hand, stromal cells have long been known to have the potential to give rise to myogenic cells, which probably accounts for their survival after engraftment into necrotic myocardium and the related improvement in function.28 However, a more specific switch toward a cardiomyogenic phenotype seems to require preexposure to 5-azacytadine before transplantation.28 This demethylating agent may cause an unregulatable expression of various genes, thereby raising clinically relevant safety issues. In the absence of 5-azacytidine, in-scar implanted stromal cells fail to express cardiac specific markers, particularly, connexin 43,29 which is critical for synchronous electromechanical coupling with host cardiomyocytes and consequently, these BM cells are unlikely to offer any advantage over skeletal myoblasts which similarly lack this junction protein.6 Alternatively, mesenchymal cells can be oriented toward a cardiomyogenic lineage by co-culturing or co-implanting them with fetal cardiomyocytes30 because a direct cell-to-cell contact is an effective means of inducing the targeted differentiation of cells which feature a plasticity potential.31 Whether the recently isolated fraction of mesenchymatous adult pluripotent cells32 could be more amenable to a cardiomyogenic differentiation in response to the sole environmental cues remains to be determined as well as its potential to functionally repopulate infarcted myocardium following transplantation.

In conclusion, transplantation of freshly aspirated, unfractonated BM into postinfarction scars fails to provide any benefit, which contrasts with its potential efficacy when implemented at an earlier stage of the injurious process. Should this observation be validated by further studies, its clinical correlate would be that BM-derived cells are best indicated in the setting of acute coronary syndromes with the more specific objective of increasing angiogenesis whereas skeletal myoblasts remain more effective cell substitutes for improving function at the late stage of chronic heart failure.

References

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