Long-Term Efficacy of Myoblast Transplantation on Regional Structure and Function After Myocardial Infarction

Saïd Ghostine, MD; Claire Carrion, MSc; Luiz César Guarita Souza, MD; Pascal Richard, MD; Patrick Bruneval, MD; Jean-Thomas Vilquin, PhD; Bruno Pouzet, MD; Ketty Schwartz, PhD; Philippe Menasché, MD, PhD; Albert Alain Hagège, MD, PhD

Background—Transplantation (Tx) of skeletal myoblasts (SM) within an infarcted myocardium improves global left ventricular (LV) function, although a direct systolic effect remains controversial.

Methods and Results—Global and regional LV functions were studied in a sheep model (n=16) of infarction before (baseline), and 4 (M4), and 12 (M12) months after in-scar injections of autologous SM or culture medium (CM). LV end-diastolic volume (EDV), ejection fraction (EF), wall motion score (WMS), and systolic myocardial velocity gradient (MVG) across the scar were measured by echocardiography with tissue Doppler imaging. Parameters were similar at baseline between groups. At M4, Tx of SM reduced the postinfarction increase in EDV (72±8 versus 105±13 mL in the CM group, P<0.05) and the decrease in EF (48±5 versus 33±3% in the CM group, P=0.006) although it improved WMS (5.4±1.2 versus 13±2.2 in the CM group, P<0.01) and SMVG (0.60±0.13 versus −0.04±0.13 seconds1 in the CM group, P<0.05). Results were similar at M12. In-scar accumulation of myotubes and SM were detected in all Tx animals up to M12, with co-expression of fast and slow isoforms of the myosin heavy chain (MHC) (30% of the fibers versus 0% in the normal skeletal muscle) and decreased collagen density (30±2% versus 73±3%, P<0.0001).

Conclusion—For up to 1 year, Tx of SM limits postinfarction EF deterioration and improves systolic scar function through colonization of fibrosis by skeletal muscle cells with expression of both MHC isoforms, which may confer to the graft the ability to withstand a cardiac-type workload. (Circulation. 2002;106[suppl I]:I-131-I-136.)

Key Words: transplantation ■ skeletal myoblasts ■ cell therapy ■ myocardial infarction

Cellular transplantation has recently emerged as a potential new treatment of ischemic cardiomyopathy.1 Experimental studies have demonstrated improvement in global left ventricular (LV) function following engraftment of cultured adult skeletal muscle stem cells (myoblasts) within an infarcted myocardium.2–6 However, a potential direct systolic effect remains controversial because the presence of gap junctions between host cardiomyocytes and grafted tissue has not been demonstrated7,8 even if previous experiments (short-term studies often in small animals) have suggested an improvement in regional function.3,4,7,9 Tissue Doppler imaging (TDI) is a recent technique10,11 that has been shown to accurately detect early changes in myocardial contractility or relaxation and is more sensitive than the changes in segmental thickness.12 The current study aims to evaluate long-term (up to 1 year) global, regional, and segmental functional effects of cultured myoblast transplantation in a sheep model of myocardial infarction using echocardiography and TDI and to correlate these changes 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.

Sheep (45 to 50 kg) were supplied by the Institut National de Recherche Agronomique, France. Under anesthesia (thiopental sodium, Pentotal, 15 mg/kg, Abbott Laboratories), and tracheal ventilation, catheterization of the circumflex coronary artery was performed using the Seldinger technique with opacification with ioxithalamate sodium (Telebrix 35, Guerbet Laboratory). Myocardial infarction was created by embolization with absorbable hemostatic gauze (Surgicel, Johnson & Johnson) under echocardiographic monitoring.

Cell Cultures

At the time of infarction, a 10-g skeletal muscle biopsy was obtained from the vastus lateralis and was finely minced. Cultures were performed during 14 days, as previously described.3 The cultures were expanded twice by trypsinization before reaching confluence. In 1 case, 1 year after implantation, culture was initiated from a small piece of myocardial tissue harvested from the cell-grafted area.

From INSERM EMI-0016, Necker-Paris V University and Department of Cardiology 1, Hôpital Européen Georges Pompidou (S.G., A.A.H.); Department of Cardiovascular Surgery B, Hôpital Bichat (L.C.G.S., B.P., P.M.); INSERM U523, Institut de Myologie, Pitié Salpêtrière (C.C., J.T.V., K.S.); INSERM 430, Hôpital Broussais (P.B.); and Department of Cardiology, Hôpital Cochin (P.R.), Paris, France.

Correspondence to Albert Alain Hagège, MD, PhD, Department of Cardiology 1, Hôpital Européen Georges Pompidou, 20 Rue Leblanc, 75015 Paris. E-mail hagege@club-internet.fr

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Following plating and expansion, the culture medium was shifted to a differentiation medium containing no beta-fibroblast growth factor and 2% of fetal bovine serum.

Cell Transplantation
Preparation of Cells
As previously described, the cells were harvested by trypsinization, washed 3 times, and concentrated in saline 0.9% supplemented with BSA (0.5%, Sigma Laboratory). Aliquots were prepared for further characterization of cell type and viability. Cells were kept on ice for transportation to the surgical room. Immediately before injection, cells were transferred to 1-mL syringes and warmed at room temperature.

Cell Characterization
At each expansion time and at the final harvest, the proportions of myogenic cells were evaluated by flow cytometry using a 5.0-MHz probe (5V2) for harmonic imaging or a mouse isotypic serum conjugated to rhodamine as a control. Following centrifugation, the cells were analyzed using a flow cytometer (FACalibur). Approximately 50,000 cells were screened and the data were expressed as a percentage of CD56 cells relative to the whole cell population; cell viability was assessed by flow cytometry using propidium iodide exclusion assay and data were expressed as the percentage of viable, nonfluorescent, cells (Cellquest software). The ability of the grafted cells to differentiate into skeletal muscle tissue was appreciated in vitro. At the time of cell harvest, an aliquot of cells was plated into Nunc 12 wells dishes and the growth medium was shifted to the differentiation medium (see above). On the formation of elongated and multinucleated structures, the cells were fixed and permeabilized using cold methanol and the presence of desmin was assessed using a monoclonal antibody (D33, 1/300, Dako, Denmark). The immunolocalization was further amplified using a secondary antibody conjugated with Cy3® (Jackson). The procedure for cytochemistry has been described elsewhere.

Intramyocardial Injections
Sheep having survived infarction were randomized into 2 groups receiving culture medium only (n=13, control group) or cells (n=11, myoblast group). Fourteen days after infarction, a lateral thoracotomy was performed under general anesthesia and 20 to 30 intramyocardial injections were performed across and around the infarcted area using a 27-gauge needle.

Ultrasonic Assessment
Acquisitions
Under general anesthesia, transthoracic echocardiography was performed using a 5.0-MHz probe (5V2) for harmonic imaging or a 3.5-Hz probe (3V2c) for TDI connected to a Sequoia 512 system (Acuson, Mountain View, CA, USA). Examinations were performed (1) at the time of infarction; (2) immediately before injections, 14 days after infarction (baseline); (3) 4 months after injections (M4) and (4) at 1 year. One parasternal LV long axis view (including the mitral and aortic valves and the apex) and 3 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 infarct, 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 end-diastolic areas traced at the mitral (A1), papillary muscles (A2) and apex (A3) levels. Left ventricular end-diastolic (LVEDV) and end-systolic (LVEFV) volumes were calculated using the simplified Simpson’s rule as (A1+A2)/(A3) and LV ejection fractions (EF) were then calculated.

Scoring of Regional Contractility
Using the 3 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 their algebraic sum.

TDI Studies
In each stored image, the posterior wall was divided into 2 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. 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 3 consecutive beats by a single experienced operator who was blinded to the treatment group.

Pathologic Assessment
Euthanasia was performed after the last ultrasonic evaluation and the hearts were explanted. Location and extent of myocardial infarctions were visually assessed. Sampling of the tissues consisted of scar surrounded by a ring of viable myocardium. The central part of the scar with the maximal length was fixed in 1/10 formalin for histology and morphometry. Small biopsies were taken out at various places and snap-frozen in isopentane cooled by nitrogen for immunological analysis, or kept fresh and sterile for culture. An additional sample was fixed in 2.5% glutaraldehyde for electron microscopy.

Histological Studies
Formalin-fixed tissues were embedded in paraffin. Sections were stained with hematoxylin-eosin for qualitative assessment. Sections were stained for collagen (C) with Sirius red and processed to morphometry using a computerized image analysis, as previously described. We measured the collagen density in 10 microscopic fields having the highest in-scar grafted cell density at ×20 magnification and this was compared with the collagen density from 10 random fields in control nongrafted scars; the percentage occupied by the grafted cells was calculated as (100−C)/C was determined after scanning the entire scar using adjacent microscopic fields at ×10 magnification and automatic measurements of the collagen after elimination of fat tissue and vessels.

Immunohistological Studies
Examinations were performed on 8-μm cryosections according to a previously described protocol. The MY-32 clone (Sigma) directed against the cardiac and skeletal slow myosin heavy chain (MHC) and the NOQ7 (Sigma) directed against the cardiac and skeletal slow MHC isoforms were used.

Electron Microscopy Studies
Tissues for electron microscopy were post-fixed in 4% osmium tetroxide.

Statistics
All data were reported as means±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 and M4 used the Student paired t test.
Results

Myocardial infarction was created in 44 animals; 20 animals were excluded because of lethal ventricular fibrillation at embolization. Thus, the study group included 24 animals. These animals were divided into groups including 13 control animals and 11 animals in the myoblast group. After randomization, 8 animals were excluded because of refractory congestive heart failure (CHF) before injections, lethal ventricular fibrillation at injections, or lethal CHF before evaluation [3/11 controls (27%), at 18 days, 1 and 4 months, 0/9 in the myoblast group (0%)]. Finally, 8 animals in each group remained available for echocardiography at M4; 13 animals (8 controls) were sacrificed at M4; 3 animals (from the myoblast group) were reanalyzed at 1 year before sacrifice.

Cell Cultures

Cultures yielded an average of 418 ± 78 million cells (150 to 750 millions) with 32 ± 7% (9 to 75%) of myogenic cells.

Functional Assessment (Table 1)

Baseline data were not significantly different between groups. At M4, heart rate (bpm) was unchanged in controls (111 ± 5 versus 111 ± 10 at baseline, \( P = 0.95 \)) although it was decreased in the myoblast group (87 ± 7 versus 120 ± 7 at baseline, \( P = 0.02 \)); the difference between the 2 groups was significant (\( P = 0.01 \)). LVEDV (mL) increased to a greater extent over time in the control animals (105 ± 13 versus 47 ± 6 at baseline, \( P = 0.003 \)) than in the myoblast group (72 ± 8 versus 43 ± 5 at baseline, \( P = 0.007 \)); LVEF (%) decreased in the control animals (33 ± 3 versus 43 ± 4 at baseline, \( P = 0.05 \)) but remained unchanged in the myoblast group (48 ± 5 versus 50 ± 2 at baseline, \( P > 0.05 \)); again, the between-group difference was significant (\( P = 0.006 \)). The WMS increased, indicating a deterioration of contractility in the control animals (13 ± 2.2 versus 8.9 ± 1.4 at baseline, \( P = 0.03 \)) but it was decreased in the myoblast group (5.4 ± 1.2 versus 7.7 ± 0.9 at baseline, \( P = 0.006 \)); the between-group difference was significant (\( P < 0.01 \)). Finally, TDI analysis showed a decrease in the systolic MVG (s⁻¹) in the control group (–0.04 ± 0.13 versus 0.4 ± 0.06 at baseline, \( P = 0.006 \)) but an increase in the myoblast group (0.60 ± 0.13 versus 0.33 ± 0.04 at baseline, \( P = 0.005 \)); the difference between the 2 groups was at the limit of significance (\( P = 0.05 \)). A similar trend was seen for the diastolic MVG (0.07 ± 0.2 versus 0.7 ± 0.3 at baseline in the control group; 0 ± 0.6 versus 3 ± 0.13 at baseline in the myoblast group, \( P = 0.07 \)). In the 3 myoblast-transplanted sheep, which were assessed at 1 year, functional results were unchanged from those recorded at 4 months.

Pathologic Assessment

Scars were always extended to the posterolateral LV wall and sizes were similar in the 2 groups (24.8 ± 6.3 cm² in controls versus 18.6 ± 5.4 cm² for the myoblast group). In the control group, scars were transmural with fibrous tissue intermingled with fat tissue (Figure 1A). In all transplanted animals, large areas of grafted cells were found within the scars (Figure 1B), which had the histological features of well-differentiated skeletal muscle cells (Figure 2) and often had a tendency to organize in parallel (Figure 2B). Neither necrosis nor inflammation were observed. The in-scar grafted area was 57 ± 4% and the collagen density decreased in the transplanted scars reached 30 ± 2% in the highest cellularity areas versus 73 ± 3% for the control animals (\( P < 0.0001 \)). In all transplanted animals, antibodies directed against the fast MHC isoform identified the presence of skeletal muscle structures surrounded by fibrotic and cardiac tissue, which were not labeled (Figure 3). Serial sections indicated also the presence of fibers, which expressed the slow isoform, as did the host cardiac tissue (Figure 3B); 30% of the skeletal muscle fibers co-expressed the slow and fast MHC isoforms, whereas this pattern was never observed in the vastus lateralis of these animals (Table 2). Using electron microscopy, the grafted cells exhibited a well-differentiated skeletal myocyte pattern (Figure 4, A and 4B) without any junctions or intercalated discs.
disks and myoblasts were identified close to the myotubes, often within a split of their basement membrane (Figure 4C). At 1 year, histological analysis revealed the persistence of viable myotubes and myoblasts within the scar; in 1 case, cultures from a biopsy of the grafted area could give rise to new myoblasts that merged into new myotubes.

Discussion
Myogenic cell grafting to regenerate the pool of contractile cells has recently emerged as a promising technique for severe ischemic systolic LV dysfunction. Skeletal myoblasts (or satellite cells) are stem cells functionally indistinguishable from embryonic myoblasts, located under the basal lamina of adult skeletal muscle and committed to multiply after injury. They are highly resistant to ischemia and possess a high potential for division in culture. Myoblasts transplanted in an infarcted myocardium remain viable for a few months, differentiate and merge into myotubes. The beneficial influence of this transplantation on global LV performance has been suggested by numerous experimental data. The magnitude of this improvement is indeed similar to that afforded by fetal cardiomyocyte transplantation and correlates with the number of injected cells. Thus, autologous skeletal myoblasts, which do not raise immunological, ethical, tumorigenesis or donor availability problems, have been chosen for the first phase I clinical trial of cellular transplantation within a post-infarction scar.

Limitations of Previous Studies
The regional effects of myoblast transplantation as well as the period of time over which its functional benefits are sustained have not yet been elucidated. Likewise, the mechanism(s) of the efficacy of the procedure still remain elusive. In vitro, myoblast grafts contract when stimulated electrically and can convert to fatigue-resistant slow-twitch fibers and undifferentiated rat skeletal myoblasts express N-cadherin and connexin 43. However, both proteins are markedly downregulated after differentiation into myotubes within infarcted scars; thus, synchronous graft contraction remains questionable. Nevertheless, recent experimental studies using either color kinesis echocardiography or sonomicrometry have suggested a systolic effect. However, as color kinesis tracks regional movement, cardiac motion or changes in cardiac load conditions may alter the signal; moreover, sonomicrometry uses changes in scar length with the cardiac cycle but does not provide a direct evaluation of wall thickening. Yet, no direct evaluation of changes in segmental function (increased thickness or myocardial velocities) has been attempted.

Findings of the Present Study
This study confirms the efficacy of our customized culture techniques to yield large numbers of autologous skeletal muscle cells. It also demonstrates that engrafted myotubes are not only identified up to 1 year after transplantation in the scar tissue, but that they have also retained their functionality because the myoblasts that they still harbor could trigger the formation of new skeletal muscle tissue on postharvest in vitro activation. The marked decrease in fibrous tissue is due in part to graft expansion but might also be the consequence of metalloproteinase secretion by the fibroblasts contaminat-
ing the injectate because fibroblast transplantation improves diastolic performance.\textsuperscript{20}

Although it is noteworthy that, to date, most experimental studies of cell transplantation have attributed the beneficial effects of the procedure to a scaffolding effect of the implanted cells resulting in limitation of scar expansion,\textsuperscript{3–7} TDI was able to demonstrate the reappearance of the physiologic transmural myocardial velocity gradient within the transplanted scar, which strongly suggests that the graft contributes locally to the systolic activity, whatever the underlying mechanism. If conventional ultrasound technique is widely used for the evaluation of regional LV function because it depicts in real-time wall motion (endocardial excursion and myocardial thickening), it is only a qualitative method and is both subjective and observer-dependent.\textsuperscript{21,22} The structural and functional heterogeneity of the transplanted myocardium, with a variable amount of grafted tissue surrounded by necrosis, further complicates this interpretation.\textsuperscript{12} TDI, which directly measures indexes of myocardial function within the myocardial wall, has been validated for the quantification of transmural contractile function and has been proven to be highly sensitive and specific for the detection of decreased myocardial function due to ischemic or cardiomyopathy processes.\textsuperscript{10–13} Whereas conventional ultrasound techniques derive their information on myocardial function from either parameters measured from the blood-myocardial boundaries or from blood-pool Doppler indexes (in that case, improved contractility may be confounded by the contraction of the adjacent myocardial segments), TDI directly measures indexes of myocardial function within the myocardial wall and more accurately reflects the intrinsic contractile properties of the targeted area.\textsuperscript{13} It allows computerized real-time quantification of endocardial and epicardial myocardial velocities by detection of consecutive phase shifts of the ultrasound signal reflected from the contracting myocardium and provides access to MVG, which is not influenced by variables other than regional contractility.\textsuperscript{11} This provides compelling evidence that the better functional outcome following transplantation involves both a limitation of scar expansion and a true increase in contractility.

However, the mechanism underlying this increase in contractility is not elucidated. The lack of connexin 43-supported gap-junctions, however, does not necessarily preclude coordinated heartbeats,\textsuperscript{23} thereby implying that cell-to-cell coupling can occur through alternate pathways. One possibility is that the stretch-activated channels present on myoblasts allow them to contract mechanically in response of the contraction of the surrounding host cardiomyocytes. However, this hypothesis needs to be confirmed by the direct recording of action potentials originating from engrafted myotubes.

A second important and novel finding of the present experiments is that the functional benefits of myoblast transplantation were still manifest at 1 year. This finding correlated with new-onset of expression of the slow myosin isoform by the engrafted fibers, which supports the influence of the surrounding tissue on the graft. The new myocardial environment of these fibers, particularly repeated stretch, might have partially driven their phenotype toward the slow-twitch patterns, thereby accounting for the ability of implanted cells to withstand a cardiac-type workload. Moreover, the demonstration up to 1 year of the presence of a viable graft correlates with the persistence of the segmental functional benefit. The presence of Z-bands suggests that the contractile apparatus of the fibers remains functional, whereas the normal contractile apparatus disappears in non-

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|}
\hline
MHC Isoform & Peripheral Skeletal Muscle Biopsy (Total—865 Fibers) & Grafted Area (Total—1563 Fibers) \\
\hline
Fast & 85\% & 50\% \\
Slow & 15\% & 20\% \\
Fast and Slow & 0\% & 30\% \\
\hline
\end{tabular}
\caption{Repartition of the Myosin Heavy Chain Isoforms in the Grafted Fibers and the Nongrafted Versus Lateralis}
\end{table}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure4.png}
\caption{Electron microscopy views (×10 000) of the scar in a grafted sheep at 1 year. (A) Presence of skeletal myotubes without junctions between adjacent grafted cells. (B) Well-differentiated grafted myotubes with normal organites with densely packed myofilaments, Z-bands, triades, and peripheral multiple nuclei. (C) Presence of a myoblast (arrow) within a split of the basement membrane of a myotube.}
\end{figure}
contractile muscular fibers, as shown with nerve degeneration in peripheral neurological disease.24

In this study, infarctions were intentionally of small size to avoid high operative mortality. Because the functional improvement after transplantation is related to the magnitude of the decrease in EF before the procedure, with little change at the highest EFs,8 an improvement in global EF was therefore not expected. Nevertheless, LVEF did not deteriorate over time in the treated group as opposed to controls, thereby supporting a beneficial effect of the transplantation on global performance. This finding, however, raises the question of the number of transplanted segments required to improve for the procedure to become clinically relevant. After revascularization of hibernating myocardium, the number of recovering segments is critical for LVEF recovery and a 20% cutoff is often cited.25 Cell transplantation might contribute to reach that critical threshold when associated to coronary revascularization. Lower mortality and heart rates in the treated group also support the beneficial effect of the procedure on LV performance.

Clinical Implications

This study demonstrates the efficacy of transplantation of cultured autologous myoblasts within an infarcted area. Differentiation into myotubes with expression of the slow myosin heavy chain isoform, which may confer cells the ability to sustain a cardiac-type workload, correlated with long-term graft viability and improved function. Reappearance of physiologic transmural velocity gradients within the injected scar areas suggests an active contribution to cardiac function, which further legitimizes the implementation of clinical trials.

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