Long-term (1 year) functional and histological results of autologous skeletal muscle cells transplantation in rat

Nawwar Al Attar\textsuperscript{a}, Claire Carrion\textsuperscript{b}, Said Ghostine\textsuperscript{c}, Isabelle Garcin\textsuperscript{b}, Jean-Thomas Vilquin\textsuperscript{b}, Albert A. Hagège\textsuperscript{c}, Philippe Menasché\textsuperscript{a,*}

\textsuperscript{a}Department of Cardiovascular Surgery and INSERM U 572, Hôpital Européen Georges Pompidou, 20, rue Leblanc, 75015 Paris, Cedex, France
\textsuperscript{b}INSERM U 523, Institut de Myologie, Hôpital Pitié-Salpêtrière, Paris, France
\textsuperscript{c}Department of Cardiology, Hôpital Européen Georges Pompidou and Faculté de Médecine Necker-Paris V, Paris, France

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Abstract

\textbf{Background:} Several studies have demonstrated the short-term benefits of autologous skeletal muscle cell transplantation on postinfarction left ventricular function. The present experiments were designed to assess the long-term effects of the procedure. \textbf{Methods and results:} Thirteen Wistar rats that had undergone skeletal muscle cell transplantation \((n=6)\) or injection of control culture medium \((n=7)\) in isofoms areas after myocardial infarction created by coronary artery ligation and survived for 1 year were functionally assessed by combining echocardiography and pressure–volume loops. At 1 year after transplantation, both contractile and relaxation indices were significantly improved in the skeletal muscle cell-grafted group compared with controls. One-year echocardiographic measurements of ejection fraction were similar to those recorded 2 months after the procedure. The stability of the functional outcome contrasted with a decrease in the number of histologically detectable skeletal myotubes over time. However, the proportion of the slow and composite (fast and slow) myosin isoforms expressed by skeletal muscle fibers still present after 1 year was greater than that found in animals sacrificed after 2 months. \textbf{Conclusion:} The functional benefits of autologous skeletal muscle cell transplantation are sustained over time and are associated with either selection, preservation or an increased expression of slow myosin heavy chain isoforms. The discrepancy between maintenance of this improvement and the decay in the engrafted myotubes suggests protective mechanisms operative from the early post-transplantation stage and possibly involving modulation of extracellular matrix remodelling or paracrinally induced maturation of putative cardiac resident stem cells.

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1. Introduction

Medical treatment offers limited promise to patients with end-stage heart failure. Even though heart transplantation is the only established radical treatment, it is fraught with several drawbacks, specifically those related to organ shortage, complications of immunosuppression and late graft vasculopathy. In this setting, cell therapy has recently emerged as a novel approach for improving severe ischaemic left ventricular (LV) dysfunction.

So far, most studies have focused on skeletal muscle cell transplantation which has been shown to provide short term beneficial effects on ventricular function in both small (rats) \([1,2]\) and large (rabbits) \([3,4]\) animal models of myocardial infarction (MI) created by coronary artery ligation or cryoinjury. However, the long-term consequences of skeletal muscle cell replacement therapy have yet to be demonstrated. The objective of the present study has been to address this issue by assessing the outcome of...
skeletal muscle cell transplantation after a 1-year follow-up in a rat model of myocardial infarction.

2. Methods

The experiments complied with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, Commission on Life Science, National Research Council, and published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Myocardial infarction model

Male Wistar rats were anesthetised with ketamine (50 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) and ventilated with an endotracheal tube. A MI was then created by ligation of the left coronary artery with a 7-0 polypropylene snare (Ethicon Somerville, NJ, USA) through a left lateral thoracotomy.

2.2. Cell culture and implantation

The tibialis anterior muscle was chosen because of easy access while its size allows the preparation of an amount of cells convenient for our experimental studies with minimal harm to the animal. At 48 h before the creation of the MI, the muscle was preconditioned using Marcain as previously described [5]. Simultaneously with the creation of MI, the tibialis anterior muscles were dissected, minced, weighed, and enzymatically dissociated using collagenase IA (2 mg/ml; Sigma, St. Louis, MO, USA) and trypsin–EDTA (0.25%, Gibco, Gaithersburg, MD, USA). The cells were grown in F12 medium (Gibco) supplemented with 15% foetal bovine serum (Hyclone, Logan, UT, USA), 1% penicillin–streptomycin (Gibco) and 5 ng/ml bFGF (Sigma). At the end of the 7–9 day culture process, the cells were harvested by trypsinisation, pelleted and delivered intramyocardially into the infarcted area in two to three sites using a 30G needle. A sample was plated for the phenotypic characterisation of myogenic cells expressing the desmin cytoskeletal filament according to a previously described cytochemistry procedure [1].

2.3. Experimental groups

Under general anaesthesia, ventricular function was assessed by echo-cardiography 7 days after infarction. The next day, rats were randomly injected through a mini-sternotomy with either 150 µl of the myogenic cell suspension (skeletal muscle cell group) or an equivalent volume of the culture medium (control group).

2.4. Functional assessment

2.4.1. Echocardiography

Echocardiographic evaluation of LV function was repeated at 2 months and 1 year from the time of transplantation according to a previously described protocol [2].

2.4.2. PV loops analysis

This assessment was performed at 1 year from transplantation using a conductance catheter (Mikro-Tip 2Fr pressure–volume catheter, Millar Instruments, Houston, TX, USA). Following the echocardiographic evaluation, the catheter was introduced through the right internal carotid artery into the left ventricle. Adequate positioning was checked by demonstration of ventricular pressure tracings on the screen. The catheter was connected to a conductance unit (MPCU-200, Millar). PV loops were analysed using iox software (EMKA Technologies, Paris, France). A fine tubing was inserted into the right internal jugular vein and used for saline injection which allowed for computation of parallel conductance. Calibration, application and analysis of the conductance catheter technique for measuring ventricular volumes has been previously described [6]. Ejection fraction (EF) was measured from the steady state PV loops. Following transient cross-clamping of the inferior vena cava, occlusion sets of PV loops recorded under respiratory arrest allowed to compute the following parameters: end-systolic pressure–volume relationship (ESPVR), preload recruitable stroke work (PRSW), maximal elastance (E\text{max}), preload-adjusted dP/ dt\text{max} (dP/dt\text{max} − EDV) and end diastolic pressure–volume relationship (EDPVR).

2.4.3. Histological assessment

After PV loop analysis, the animals were sacrificed. The hearts were snap-frozen in nitrogen-cooled isopentane. Standard histological studies were carried out on cryostat sections (hematoxylin–eosin staining). The skeletal muscle tissue (myotubes and small muscle fibers) was specifically detected with a monoclonal antibody directed on serial sections separated by 8 µm each, against the fast (skeletal) myosin heavy chain (MHC) isoform (clone MY32, Sigma). The expression of the slow MHC isoform expressed both in heart and some skeletal muscle cells was assessed using a monoclonal antibody (clone NQ7, Sigma). Following identification of myotubes at the lowest magnification, the number of cells positive for the fast, slow, or both MHC isoforms, was counted manually on high-magnification micrographs on the heart section containing the highest number of skeletal muscle fibers.

2.5. Data analysis

Functional studies were performed in a blinded fashion. Between-group comparisons of EFs derived from echocardiography and PV loops were done by the Wilcoxon
test. Mann–Whitney U tests were used to study functional variables within the same group. Results are expressed as mean±S.E.M. The critical α level was set at $P<0.05$.

3. Results

3.1. Cell cultures and transplantations

Within 1 week, the cell culture procedure allowed to grow and inject $9.4±3.3\times10^6$ cells, of which an average of 31.5% was identified as skeletal muscle cells by a positive staining for desmin.

Thirty-two rats out fifty-seven rats subjected to MI survived the procedure. During follow-up, thirteen rats were sacrificed at 2 months for histological evaluation; eight of them had received skeletal muscle cells. An additional six died (four controls and two skeletal muscle cells) during the follow-up period, leaving a total study population of thirteen rats for functional evaluation (seven control rats and six skeletal muscle cell-transplanted rats) with an average weight of 665.15 g 1 year after transplantation.

3.2. Functional results

3.2.1. Echocardiography

Baseline studies performed after MI showed comparable LVEFs in the two groups (28±3% in controls and 27±4% in skeletal muscle cell-transplanted rats, $P=0.47$). Two months after transplantation, there was a significant improvement of EF in the skeletal muscle cell group (42±6% vs. 20±2% in controls, $P=0.002$). At the 1-year post-transplant study point, an improvement of LVEF of similar magnitude was still observed in the skeletal muscle cell group: 45±12 vs. 26±7% in controls, $P=0.015$ (Fig. 1A).

3.2.2. Pressure–volume loops

LVEF measured by PV loops was not significantly different from that derived from echocardiography. This correlation was acceptable for the control group (32±9 vs. 26±7%, respectively, $P=0.06$) and still better in the

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Fig. 1. Functional effects of autologous skeletal skeletal muscle cell transplantation. (A) Echocardiographic changes of left ventricular ejection fraction over time. (B) Representative tracings of pressure volume loops in a control (right panel) and a myoblast skeletal muscle cell-transplanted (left panel) heart. ESPVR, end systolic pressure–volume relationship; rvu, relative volume units.
skeletal muscle cell group (47±12 vs. 45±12%, respectively, \( P=0.75 \)).

PV loops analysis demonstrated better preservation of baseline indices comparing to controls (ESPVR: 14.87±1.24 vs. 7.40±1.30, \( P=0.043 \); PRSW: 50.8±1.4 vs. 35.7±2.8 mmHg, \( P=0.004 \); \( E_{\text{max}} \): 17.74±1.37 vs. 5.72±1.12 mmHg \( \mu \text{l}^{-1} \text{P}<0.0001; \ dP/dT_{\text{max}} \)-EDV: 409.68±114.14 vs. 100.92±13.70 mmHg s\(^{-1} \mu \text{g}^{-1}, \ P=0.0043 \), respectively). Representative tracings of ESPVR clearly show that the heart in the control group is dilated with a rightward shift of the curve whereas in the skeletal muscle cell-grafted heart, the slope of the regression line of the left upper points of the loop are shifted to the left, thereby indicating a better inotropism (Fig. 1B). Likewise, the significantly smaller value of EDPVR in the skeletal muscle cell group (0.17±0.02) compared with the controls (1.82±0.11, \( P=0.003 \)) reflects a reduced ventricular stiffness following skeletal muscle cell implantation.

3.2.3. Immunohistopathology

Hematoxylin–eosin staining revealed in-scar myotubes (Fig. 2, arrowheads) with surrounding host cardiac tissue 2 months (data not shown) and 12 months (Fig. 2a, upper part of the picture) after transplantation. The expression of fast skeletal MHC (Fig. 2b) provides direct evidence for skeletal muscle cell engraftment into the myocardial scar. Interestingly, in these serial sections, engrafted cells also expressed a slow MHC (Fig. 2c) isoform, which is also constitutively expressed by nearby native cardiac muscle fibers (asterisk).

At 2 months from transplantation, skeletal muscle fibers were present in all cell-injected hearts (1041±796 fibers expressing either isoform of MHC). The proportions of MHC isoforms were: fast, 47%; slow, 23%; both fast and slow, 30%. By contrast, at 12 months, skeletal muscle was present in half of the cell-injected hearts (three out of six). The number of fibers was less important in these three rats than that observed 2 months after injection (266±215

Fig. 2. Histological analysis—serial sections are presented. (a) Hematoxylin–eosin staining identifies the remaining cardiac tissue at the border of the infarcted area (top) and elongated, thin skeletal muscle fibers and myotubes within the scar tissue (medium and bottom, arrowhead). (b) Skeletal muscle tissue (arrowhead) is specifically, strongly stained by an antibody directed against the fast MHC isoform (clone MY32), while the cardiac tissue is stained at background level. (c) Both skeletal (arrowhead) and cardiac (star) tissues are labelled by the antibody directed against the slow MHC isoform (clone NOQ-7). Taken together, (b) and (c) indicate that the skeletal muscle fibers may express either the fast, the slow or both MHC isoforms. Scale bar in (a), 40 \( \mu \text{m} \).
fibers expressing either isoform of MHC). The proportions of MHC isoform were then fast, 23%; slow, 34%; and both fast and slow, 43%. This indicates a change in the proportion of MHC isoforms towards a slow-type pattern in the remaining muscle fibers over time. With the caveat that the sample size is small, analysis of individual data failed to detect a correlation between the initial number of injected skeletal muscle cells and the number of fibers that could still be identified 1 year later. Likewise, the magnitude of the functional improvement seen at the 1-year follow-up time point was not clearly related to the number of surviving muscle fibers.

4. Discussion

Our main finding is that skeletal muscle cell transplantation provides a sustained improvement of both contractility and relaxation over a 1-year follow-up period. This improvement, which correlates in half of the transplanted rats with the persistent engraftment of skeletal muscle cells, is demonstrated by echocardiography and supported by PV loops analysis.

4.1. Analysis of systolic function

Improved systolic function in transplanted rats is reflected by the increased EFs calculated from both echocardiography (45±12 vs. 26±7%, \(P=0.015\)) and PV loops (47±11 vs. 32±9%, \(P=0.025\)) and the consistency of these data is strengthened by the observation that measurements yielded by the two methods were satisfactorily correlated in both the control and transplanted groups. This improved contractility after transplantation is also demonstrated by analysis of PV loops obtained by reducing preload through temporary occlusion of the inferior vena cava. These occlusion loops demonstrate that ESPVR and its slope, \(E_{\text{max}}\), are shifted to the left in the skeletal muscle cell group (\(P=0.004\) and \(P<0.0001\), respectively). These two parameters are considered more reliable than EF for assessing systolic function [7]. Additionally, \(\Delta P/\Delta V_{\text{max}}\) – EDV and PRSW also improved in the treated group. These indices are accurate markers of ventricular contractility and are relatively independent of the loading conditions of the heart [8,9].

This improved contractility after transplantation is consistent with short-term data previously published in the rabbit [3,4] and the rat [1,2,5]. The present findings extend these observations by showing not only that the indices of contractility observed in transplanted rats are significantly superior to those of controls, but also that this improvement persists over time, as echocardiographic data recorded 1 year after transplantation were unchanged from 2-month values (Fig. 1).

Likewise, the histological analyses confirm the consistent presence of typical myotubes in the transplanted hearts at the 2-month follow-up study point, thereby supporting the likely causal relationship between initial skeletal muscle cell engraftment and improvement of ventricular function. This link had already been noted [3,4] in studies where a functional benefit was only observed in hearts in which the grafted myoblasts could be successfully identified within a few weeks of transplantation, thereby indicating that the initial presence of the skeletal muscle fibers is a critical determinant of the functional outcome.

One could argue that skeletal muscle cells only represented a limited proportion of the total cell yield which was transplanted and that the observed benefits on function could be due to other cell types present in the injectate. Although we did not perform a thorough phenotypic characterisation of these other cell lineages to spare the already limited number of available cells, it is expected that they mostly comprised fibroblasts. A comparative functional assessment of transplanted autologous skeletal myoblasts versus dermal fibroblasts into chronically injured rabbit heart by micromanometry and sonomicrometry has showed that whereas fibroblast transfer improved diastolic performance, only myoblasts could increase systolic function in the damaged region, supporting the role of myogenic cells in augmenting contraction [10]. Likewise, Sakai et al. demonstrated that foetal cardiomocytes transplanted into myocardial scar provided greater contractility than foetal smooth muscle cells or foetal fibroblasts thereby providing additional evidence that the intrinsic contractile properties of the implanted cells are a prerequisite for systolic function to improve [11].

Strikingly, in the present study, whereas we always observed the presence of skeletal muscle tissue in the injected hearts 2 months after transplantation, we failed to identify it in half of the hearts 1 year after grafting. Moreover, the mean number of fibers was smaller after 12 months than after 2 months after implantation. Interestingly, surviving myotubes of the transplanted hearts coexpressed fast and slow myosin isoforms. The increased time-averaged proportion of myosin isoforms expressing a slow or composite (fast and slow) pattern in skeletal muscle cell-injected hearts raises two hypotheses which are not mutually exclusive. First, by analogy with what has been reported with dynamic cardiomyoplasty, the ‘conditioning’ of skeletal muscle by the electromechanical activity of the surrounding cardiac tissue might switch the phenotype of implanted cells towards a slow cardiac-type myosin expression. This fatigue-resistant pattern would, in turn, account for the ability of the engrafted cells to sustain a cardiac-type workload over a prolonged period of time. Second, in the context of a cardiac environment, the fibers expressing at least the slow isoform would be positively selected at the expense of the fast ones which would then progressively disappear. This would account for the lower total number of fibers observed 1 year after transplantation, as compared with 2-month values.
The mechanism of improved systolic function remains elusive. Differentiation into myotubes is accompanied by a loss of expression of the junction proteins (connexin-43 and N-cadherin) [12] and the subsequent failure of transplanted skeletal muscle cells to establish gap junctions with host cardiomyocytes belittles an electromechanical coupling theory. However, the finding that conditional knockout mice for connexin-43, still demonstrate normal impulse propagation and LV function [13], suggests that coordinated heartbeats might occur through connexin-43-independent pathways. These alternate mechanisms could involve mechanical contractions of engrafted skeletal muscle cells elicited by those of the neighboring cardiomyocytes or electrical field effects whereby currents generated by host cardiomyocytes are directly channeled through cell membranes and subsequently trigger action potentials in excitable skeletal muscle cells. So far, however, these hypotheses have not been demonstrated. Indeed, the finding that the functional benefits of skeletal muscle cell transplantation were sustained over time in spite of the decreased number of detectable myotubes also raises a different hypothesis whereby protection would be triggered by the initial presence of the engrafted skeletal muscle cells and involve long-standing paracrine effects on extracellular matrix remodelling [14] or maturation of resident cardiac stem cells [15] into cardiomyocytes by myoblast-derived growth factors [16].

4.2. Analysis of diastolic function

Previous studies have shown that cell transplantation primarily improved diastolic function [3,4,9] through a ‘scaffolding’ effect of the engrafted cells which would limit scar expansion and the associated LV remodelling. The present data support these findings by showing a leftward shift of EDPVR in the skeletal muscle cell group (P = 0.003), reflecting a reduction of dynamic rigidity [17]. This observation is consistent with the previously mentioned hypothesis of a remodelling effect persisting beyond the decrease in the number of skeletal muscle fibers.

4.3. Limitations of the study

One limitation of this study is the small sample size (thirteen rats) resulting from the mortality that occurred after creation of infarctions and throughout the 12-month follow-up period. However, this should not have altered the essence of the conclusions. Likewise, one could also argue that pressure–volume loop analysis was performed only at 1 year without a pretransplantation assessment. This was due to our concern of not increasing the procedural mortality that would have resulted from an invasive left heart catheterisation between infarction and cell transplantation. However, baseline echocardiographic data were similar in the two groups, which tends to validate their comparability before transplantation and thus the meaningfulness of differences in postgrafting pressure–volume loop data.

In conclusion, at 1 year from autologous skeletal muscle cell transplantation in an ischaemic cardiomyopathy model, the early postprocedural improvement of LV function is sustained and involves both systolic and diastolic functions. It is associated with either the preservation of fibers expressing at least the slow myosin heavy chain, or the increased expression of this isofom, thus resulting in an increased proportion of fibers expressing it. Given the lower number of skeletal muscle fibers present 1 year after transplantation, a limitation of scar remodelling induced early after grafting by the in situ formation of skeletal muscle tissue or a skeletal muscle cell-derived paracrine differentiation of putative cardiac stem cells might be primarily responsible for the long-term preservation of functional improvement.

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