Incorporation and integration of implanted myogenic and stem cells into native myocardial fibers: Anatomic basis for functional improvements

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Background: Myoblasts and stem cells implanted into myocardium can differentiate into myocytes and may functionally improve impaired ventricles. For implanted cells to actually contribute to the synchronous contractions of the heart, however, they must be anatomically integrated with the existing native myocardial fibers.

Methods: Isogenic Lewis rats were used as donors and recipients to simulate clinical autotransplantation. Either skeletal myoblasts or marrow stromal stem cells were isolated from donors, culture expanded, and labeled with 4′,6-diamidino-2-phenylindole (4′,6-diamidino-2-phenylindole). Labeled cells were then injected into the myocardium of recipients. At intervals specimens were obtained, sectioned, and stained with hematoxylin and eosin and immunohistochemically against connexin-43 to demonstrate gap junctions (intercalated discs).

Results: At 1 week the labeled cells were still undifferentiated, but early expression of connexin-43 could be detected at contact points between the implanted cells and the native myocytes. By 4 to 6 weeks, labeled, fully differentiated myocytes could be seen to interconnect among themselves and with native cardiomyocytes by means of intercalated discs. In sections parallel to the myofibers, full integration of new labeled myocytes with the native myofiber cells could be observed. Furthermore, the labeled myofibers were in parallel with the native, unlabeled fibers. We postulate that such supracellular structural integration was enhanced by fiber stretching during cardiac contractions, sending signals for cellular reorientation and incorporation, in which the cytoskeletal system may play an important role.

Conclusion: We conclude that implanted precursor cells can be integrated into native myocardial structure so as to contribute to myocardial function. Direct cell-to-cell contact seems to be an important signaling mechanism, which has implications for cellular implantation strategies.

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The aim of cell replacement therapy for heart failure is to replace cardiomyocytes irreversibly lost to necrosis and apoptosis. Various donor cell sources have been studied, including fetal cardiomyocytes,[1] myoblasts,[2-7] and embryonic and adult stem cells.[8-10]

Use of autologous myoblasts (satellite cells)[6] and marrow stromal stem cells (MSCs)[9] has clinical advantages, because these cells are readily available for every patient and their use does not require immunosuppression. Our earlier studies and those of others have confirmed that these cells are able to survive and undergo myogenic differentiation on implantation into myocardium.[11] Although the ultimate phenotypic expression of implanted skeletal myoblasts remains controversial at this time,[4] there is strong evidence that MSCs can be

induced to express a cardiomyocytic phenotype, both in vitro[8] and in vivo.[9] Such differentiation of MSCs, which are now known to be pluripotent stem cells residing in the bone marrow of both immature and mature subjects, has been investigated and elucidated at molecular and cellular levels.[12] To achieve the clinical goal of using these cells to provide new contractile muscle capable of assisting the ventricular function of a failing heart, however, we need additional evidence that such cells can in fact be incorporated and integrated into the supracellular anatomic architecture of the myocardium.

Unlike other striated muscles, the myocardium is uniquely structured to achieve synchronous, all-or-none contractions in response to a depolarizing electric signal. Thus the cardiomyocytes are interconnected by specialized cell-cell junctions, histologically known as *intercalated discs,* to form syncytial fibers. These fiber bundles[13] are arranged in complex layers of circular, oblique, and longitudinal orientations to form the ventricular wall, optimized to perform its physiologic functions. This report explores the feasibility of supracellular integration of the neocardiomyocytes and myofibers derived from the implanted myoblasts and stem cells by extending and reappraising the observations made in our recent studies.[6,9] On the basis of these findings and the existing information on cellular mechanotransduction and cytoskeletal function,[14] we further propose a mechanistic hypothesis as to how such integration may take place, thus providing an anatomic basis for their participation in improving ventricular function in damaged hearts.

Methods

Animals

Isogenic male Lewis rats weighing 200 to 250 g (Charles River Laboratories, Laprairie, Quebec, Canada) were used as donors and recipients to simulate clinical autologous implants. Animals received humane care in compliance with the "Guide to the Care and Use of Experimental Animals" of the Canadian Council on Animal Care.

Isolation and Culture of Skeletal Myoblast Cells

After overdose with pentobarbital, the skeletal muscles of both hind quarters of adult rats were excised. As described previously,[6] the muscle was rinsed with 70% ethanol and then with 15 mL Hanks balanced salt solution (Life Technologies, Inc, Rockville, Md). It was then minced with scissors. After addition of 50 mL Hanks balanced salt solution, muscle fragments were sedimented at 2000 rpm for 2 minutes. The supernatant was discarded. The specimen was then incubated in 100 mL of 1% collagenase (Sigma, St Louis, Mo) and 0.2% type 1-S hyaluronidase in M199 (Life Technologies), supplemented with 5000-U/mL penicillin and 5000-μg/mL streptomycin (Life Technologies), for 60 minutes at 37°C in a shaker bath. Remaining muscle mass was then spun at 2000 rpm for 2 minutes. The sedimented muscle fragments were then incubated with 50 mL of 1% pronase solution (Sigma) for 30 minutes at 37°C to release myogenic cells. Fetal bovine serum (Life Technologies) was added to the total supernatant to halt further enzymatic cleavage processes. Isolated myoblast cells were plated on 60 mm polystyrene tissue culture dishes. (Corning Incorporated, Corning, NY) at a density of 1×10^6 cells per culture dish. Growth medium, each 100 mL composed of 82 mL of M199, 7.4 mL of minimal essential medium (Sigma), 10 mL of fetal bovine serum, 5000 U/mL penicillin, 5000 μg/mL of streptomycin, 250 μL of amphotericin B, and 40 μL of gentamicin, was replaced every 24 to 48 hours. Culture dishes seeded with myogenic cells were maintained in a 37°C humidified atmosphere (95% air with 5% carbon dioxide) and passaged every 48 hours. Myoblasts were subjected to a 30-second period of treatment with trypsin (Life Technologies) diluted 10 times in M199 to facilitate detachment of cells from the tissue culture dishes.

Isolation and Culture of MSCs

Isolation and primary culture of MSCs were performed according to the method of Caplan.[15] As described before,[9] MSCs were isolated from the femoral and tibial bones of donor rats. These cells in 10 mL of complete medium were then introduced into tissue culture dishes. Medium was completely replaced every 3 days, and the nonadherent cells were discarded. To prevent the MSCs from differentiating or slowing their rate of division, each primary culture was replated (first passaged) to three new plates when the cell density within colonies became 80% to 90% confluent, approximately 2 weeks after seeding. After the twice-passaged cells became nearly confluent, they were harvested, labeled with 4′,6-diamidino-2-phenylindole (DAPI),[16] and used for the implantation experiments described here. The cells were cultured in complete medium consisting of Dulbecco modified Eagle medium containing selected lots of 10% fetal calf serum and antibiotics (100-U/mL penicillin G, 100-μg/mL streptomycin, and 0.25-μg/mL amphotericin B; Life Technologies) at 37°C in a humidified atmosphere with 5% carbon dioxide.

Skeletal Myoblast and MSC Labeling

Sterile DAPI solution was added to the culture medium on the day of implantation at final concentrations of 200 μg/mL for myoblasts and 50 μg/mL for MSCs. The dye was allowed to remain in the culture dishes for 30 minutes. The cells (either myoblasts or MSCs) were rinsed 6 times in Hanks balanced salt solution to remove all excess, unbound DAPI. Myoblasts and MSCs were collected (approximately 1×10^6 cells for each implantation) and resuspended in minimal volume of serum-free Dulbecco modified Eagle medium. At this point they were ready for implantation into the myocardium.

Skeletal Myoblast and MSC Implantation

Isogenic male Lewis rats weighing 200 to 250 g served as the recipients of the donor cells. Anesthesia was induced, the animal was intubated, and a left lateral thoracotomy was performed in the fourth intercostal space. Under direct vision, 1×10^6 labeled cells (skeletal myoblasts or MSCs) were injected into the left ventricular wall with a 28-gauge needle. An 8-0 Prolene suture (Ethicon Ltd, Peterborough, Ontario, Canada) was used to mark the injection track. The thoracotomy was closed, and the animal was returned to the animal care facility.

Harvesting of Hearts

The rats were taken for their final experiments at the following intervals after the implantation: 1 week (n = 4 rats), 4 weeks (n = 4 rats), and 6 weeks (n = 6 rats). After overdose with pentobarbital, the hearts were exposed and injected with 100 mL saline solution (0.9%) through the posterior wall of the left ventricle, avoiding the transplanted area, then cryoembedded after protection with 20% sucrose in phosphate-buffered saline solution or embedded in paraffin after being perfusion fixed with 2% paraformaldehyde in phosphate-buffered saline solution.

Histologic and Immunohistochemical Examinations

The lateral wall of left ventricle was isolated from the remainder of the heart. Frozen and paraffin sections 6 μm in thickness were collected across a set of glass slides to ensure that different stains could be carried out on successive sections of tissue cut through the implantation area. One of the sections was mounted without stain for location of the DAPI-labeled donor cells under a fluorescence microscope. A successive section was stained with hematoxylin and eosin to depict nuclei, cytoplasm, and connective tissue. Other sections were selected for immunostaining of connexin-43 with rabbit polyclonal antibody (Zymed Laboratories Inc, South San Francisco, Calif). Sections were incubated in primary antibodies overnight at 4°C. Detection used secondary anti-connexin-43 antibodies conjugated with fluorescein. Combined DAPI and fluorescein images were made with simultaneous excitation filter under a reflected light fluorescence microscope (BX-FLA; Olympus America Inc, Melville, NY). Digital images, transferred to a computer equipped with Image Pro software (Media Cybernetics, LP, Silver Spring, Md), were subsequently printed.

Results

The labeling efficiency of cultured myoblasts and MSCs with DAPI approached 100% (Figure 1, *A* and *D*, respectively). The operative mortality in our series was less than 5%.

In the myoblast implantation series, the implanted fluorescent myoblasts appeared elongated 1 week after implantation (Figure 1, *B*). Four weeks after implantation, the implanted myoblasts formed DAPI-positive myofibers. Conspicuously, these fibers were not randomly oriented. The labeled myofibers had aligned themselves in parallel with each other and with unlabeled native cardiac myofibers (Figure 1, *C*).

In the MSC implantation series, at 6 weeks the cells in the cardiac myofibers clearly expressed connexin-43 along the axial junctions between DAPI-labeled (implanted) cells and neighboring unlabeled cells (native cardiomyocytes; Figure 1, *E*). The implanted cells had changed shape, aligned in parallel to the native cardiomyocytes (Figure 1, *F, top*), and fully integrated into the cardiac myofibers. In fact, these cardiac myofibers containing new labeled cardiomyocytes appeared completely normal under hematoxylin and eosin staining (Figure 1, *F, bottom*).

Discussion

Electromechanical coupling of myocardial cells is necessary for the myocardial syncytium to contract as a unit. Myocardial cells are connected by specialized cell-cell junctions, the intercalated discs, which allow electrical and mechanical continuity among cells. Intercalated discs consist of gap junctions (connexin-43 is the major gap junction protein[17] responsible for electrical coupling), fascia adherens junctions (N-cadherin[18] is the major protein responsible for mechanical coupling), and desmosomes. Hexamers of connexin-43 allow exchange of ions and small molecules across the membranes of adjacent cells. Recently, Reinecke and associates[19] reported electromechanical coupling between cardiomyocytes cocultured with skeletal myotubes. They demonstrated expression of cadherin and connexin-43 at junctions between cultured myotubes and cardiomyocytes. The junctions formed were responsible for electrical stimulation and contraction of myotubes. These skeletal myotube-cardiomyocyte junctions transferred calcium and dye across the membranes. These junctional proteins appeared to be downwardly regulated after intramyocardial implantation, although the reason for this was not clear. However, Robinson and colleagues[20] showed in vivo that C2C12 myoblasts engrafted into murine hearts after arterial injection formed intercellular connections similar in ultrastructure to gap junctions. These junctions were rich in connexin-43, fascia adherens, and desmosomes, signifying electromechanical coupling of adjoining C2C12-native myocardial cells.

On the basis of our findings and the data reported by these other investigators,[19,20] we propose the hypothesis that implanted cells express specific proteins to form gap junctions on contacting the host myocardium. These cell-cell junctions allow the implanted cells to communicate electrically with the rest of the myocardial syncytium and expose the implants to mechanical stretch of cardiac contraction generated by the adjacent cells. We posit that such mechanical signals cause implanted cells to alter their shape, migrate,[14] and integrate into existing myofibers (Figure 2). The orientation of new myofibers is guided by the vector of the contractions of adjacent fibers, such that they also align in parallel with other fibers in that particular layer of the myocardium.[14]

Mechanical force has been shown to function as an environmental cue to trigger phenotypic changes in many cell types, such as vascular smooth muscle and endothelial cells. For example, Kanda and Matsuda[21] demonstrated that exposure of cultured smooth muscle cells to periodic stretch induced alignment of cells parallel to the direction of stretch and increased the abundance of myofilaments. Wilson and associates[22] have suggested that mechanical strain is sensed by integrins and specific matrix proteins in vascular smooth muscle. Such understandings of the capability of cells for

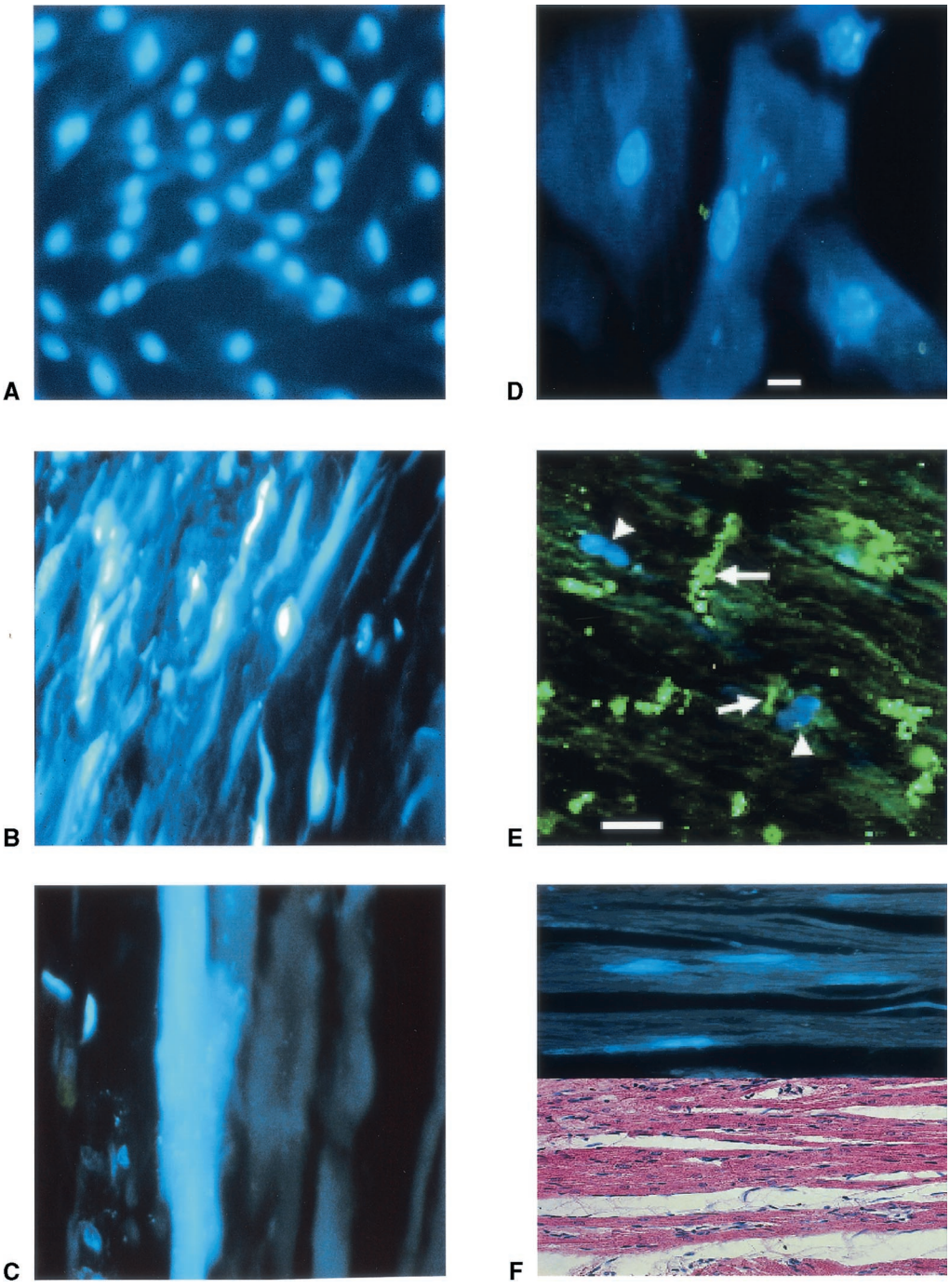

Figure 1. A, Cultured skeletal myoblasts stained with DAPI *(blue fluorescence).* Note that virtually 100% of cultured cells are labeled.[6] B, Labeled cells within host myocardium 1 week after myoblast implantation. Note undifferentiated appearance of cells.[6] C, Labeled myoblasts 4 weeks after implantation have developed into labeled myofibers. Note that these new fibers are in parallel with adjacent native, nonfluorescent cardiac myofibers. D, Cultured MSCs stained with DAPI *(blue fluorescence).* Note that virtually 100% of cultured cells are labeled. E, Connexin-43 expression of MSCs in specimen 6 weeks after implantation. Combined DAPI *(blue fluorescence)* and connexin-43 *(green fluorescence)* image. Anti-connexin-43 immunofluorescence is elicited by using fluorescein-conjugated secondary antibody. Positive connexin-43 staining *(arrows)* is found at interface between DAPI-labeled cells *(arrowheads)* and neighboring unlabeled cells. F, *Top,* DAPI-labeled cells are aligned and structurally integrated into native cardiomyofibers; native cardiomyocytes in these fibers are not labeled. *Bottom,* consecutive section stained with hematoxylin and eosin. Note that implanted cells are indistinguishable from native cells.

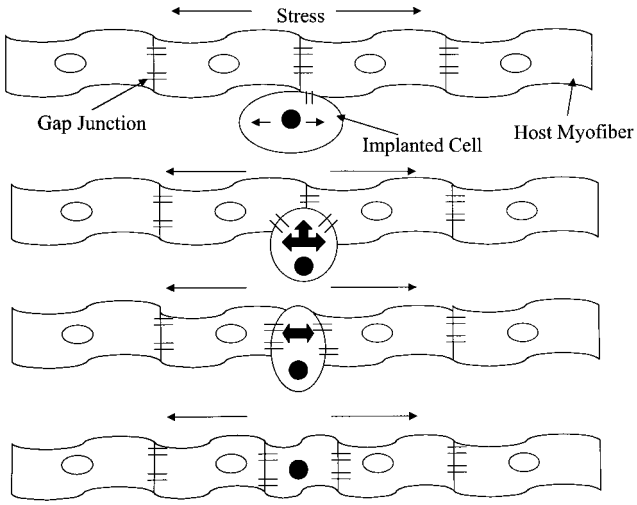

Figure 2. Schematic scenario of integration of neocardiomyocyte into native cardiac myofiber. See text for explanations.

autoassembly form the basis for culturing cells in a "bioreactor"[23] that reproduces mechanical pulsation in vitro for tissue engineering of artificial arteries. In this sense the integration in the myocardium can be seen as an autoassembly of the implanted cells, with the contractile native myocardium serving as an in vivo bio-reactor, providing the signals needed for the supracellular incorporation that we observed.

Endothelial cells exposed to steady flow likewise realign themselves in the direction of flow, a process driven by reorganization of the cytoskeleton, as demonstrated both in vitro[24]and in vivo.[25] In response to mechanical strain, actin microfilaments reorganize themselves from a banding pattern around the periphery of the cell to a series of long, parallel fibers along the long axis of the aligned cell. Cytoskeletal components F-actin and vimentin-rich intermediate filaments both connect to membrane proteins (integrins) and undergo alignment with unidirectional flow. Thus the mechanical force sensed by the extracellular matrix is transmitted intracellularly by integrins to the cytoskeleton, which leads to a cellular morphologic change. Although specific data in this regard are lacking for cardiomyocytes, we suspect a similar mechanism is involved. The mechanical stress of myocardial contraction is transmitted by the cytoskeleton to the nucleus of the implanted cell, which leads to migration, changes in the cellular shape, and integration with the existing myofibers (Figure 1, *F*). The parallel alignment of new myofibers, as seen in Figure 1, *C*, can also be orchestrated by the reorganization and function of the cellular cytoskeletal system.

From this viewpoint, it is rather perplexing that myoblasts and MSCs implanted within a myocardial scar have been reported to be able to contribute to the diastolic and systolic functions of the hearts. In these studies the cells were injected into the center of a scar caused by cryoinjury or coronary artery ligation. Although these cells seemed to undergo myogenic differentiation, they did not appear to connect and integrate with native cardiac fibers and were without evidence of gap junction formation. How such cells can contribute to systolic function and improve systolic work index[7] is not clear. Some data obtained with an isovolumic Langendorff preparation may be questioned, because the investigators used resting ventricular balloon volumes to represent preloads in constructing a Frank-Starling curve, despite the fact the cardiac sizes were significantly different between their comparison groups.[10] The hearts that received cell implants seem to be smaller than control hearts, perhaps because of reduced scar expansion and ventricular remodeling. Curiously, even implantation of smooth muscle cells has been reported to reduce cardiac dilatation.[26] Such observations have led to the suggestion that presence of such cells within the scar changes the physical property of the scar tissue, exerting a shielding effect to reduce scar expansion.[11] This explanation, however, may not be consistent with a report that cell implantation in fact improved diastolic compliance of the scar tissue, as measured with ultrasonic microcrystals placed across the scar tissue.[7] Thus there remains uncertainty regarding the reliability and mechanisms of putative improvement in cardiac function after cell implantation inside myocardial scars.

Study Limitations

In this kind of study, the technique of cell labeling used is of paramount importance, because it is crucial for differentiating myocytes derived from the implanted cells from those of the native myocardium. Although DAPI, used in this study for cell labeling, had been used and reported on by earlier investigators,[27,28] there were some concerns regarding its specificity, because the labeled cells might release DAPI molecules on their death after implantation, and leaked DAPI could then label the native myocytes in vivo to give false-positive results. Our own validation study to exclude this possibility has previously been published.[6] We tested the worst case scenario by directly injecting DAPI into the myocardium, and we confirmed that even with massive doses relative to that which might leak out from dead cells, DAPI failed to stain any native cells in vivo. We postulate that this is due to the rapid washout of free DAPI molecules in vivo, because the established labeling protocol in vitro requires incubation of these cells with DAPI for 30 to 60 minutes. Thus the limitation of using DAPI is not its possible false-positive results, but rather the risk of false-negative results if the implanted cells had divided and multiplied many times before terminal differentiation. If this were the case, the progeny cells might not be labeled, giving false-negative results that could affect the quantita-

tive results. We did not attempt quantitative analysis of our data, which was a limitation of this study.

The major advantages of DAPI relative to other techniques are that its labeling efficiency is virtually 100% and it is not toxic to the cells. It is also known that DAPI has greatest affinity to bind the regions of DNA rich in adenine-thymine pairs, but in high concentrations it can also bind to tubulin and microtubules in the cytoplasm.[29] Because in our myoblast implant experiments we used 200 μg/mL of DAPI, both the nuclei and cytoplasm were stained (Figure 1, *B* and *C*). For the MSC experiments, however, the DAPI used was 50 μg/mL, and thus only the nuclei were fluorescent (Figure 1, *E* and *F*). The discrepancy in the DAPI concentrations used is a reflection of our refinement in the labeling technique as we studied MSCs after our earlier experience with the myoblasts. This limitation does not affect the validity of our findings. Further investigations, such as correlating our observations with ultrastructures and functional data, are desirable in future studies.

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Discussion

Dr Alain Carpentier *(Paris, France).* I enjoyed this presentation, particularly the investigation of whether muscular cells are more appropriate for this type of cellular cardiomyoplasty. The ultimate goal of cellular cardiomyoplasty is to enhance contractility of the myocardium. To do so, you must demonstrate not only the survival of the implanted cells but also the transformation of the metabolism of these cells from glycolytic metabolism to oxidative metabolism. Were you able to determine that by using a specific antibody, and what did you find?

Dr Chedrawy. We have not actually looked at the metabolic aspects of cellular cardiomyoplasty. The study focused solely on the structural aspects, in hopes of providing an anatomic basis for the functional improvements that have been described. I agree that this is something to be looked into in further investigations.

Sir Magdi Yacoub *(London, United Kingdom).* I enjoyed your presentation. I have several questions. First, did you select your stem cells by flow cytometry or selecting lin negative, for example? Second, when you looked at the cells, did you look for cardiomyocyte-specific transcription factors, such as MIF-2 or GATA-4? For these myocytes or myogenic cells to function, especially if they are derived from bone marrow, they should have cardiac-specific sarcomeric proteins. Did you find, for example, troponin I or other myocardial-specific sarcomeric isoforms, because that would be essential? Finally, what do you think is the

origin of your connexin-43? Is it from the native cardiomyocytes or from the transplanted cells?

Dr Chedrawy. With respect to the first question, the MSCs were isolated according to the method of Caplan, in which the marrow was harvested from the long bones of the rats, the cells were plated on a culture dish, and the supernatant was discarded. All the cells that adhered to the culture dish, which is the distinguishing property of these cells, were used for the MSC implantation.

With respect to your second question, we did not actually look at MIF-2 as a transcription factor. We looked strictly at the implantation process and the anatomic structural integration.

With respect to your third question, we did look at cardiac-specific contractile proteins. In our case, sarcomeric myosin heavy chain protein was expressed by these implanted MSCs. We did not look at troponin I in particular.

With respect to your fourth question regarding connexin-43 expression, I am not really sure whether the MSCs themselves would express connexin-43. However, we know from in vitro studies by Dr Charles Murray's group that coculture of skeletal myotubes with cardiac cells leads to coexpression of connexin-43. So perhaps some similar mechanism is operating in this case, although I do not know for sure.

Dr Robert C. Robbins *(Stanford, Calif).* I believe that the title of your article is a bit misleading. These really are not stem cells, or at least you have not proved that they are stem cells. I probably missed it, but did you create any ischemia or any injury to the myocardium?

Dr Chedrawy. With respect to your first point, about calling the MSCs stem cells, the pluripotent nature of these MSCs is being elucidated on a monthly basis, and more and more tissues of mesenchymal, ectodermal, and neurodermal origin are being harvested from these stem cells. That is why we refer to them as stem cells and not as mesenchymal tissue origin cells.

With respect to your second concern, we actually injected all these cells into normal myocardium. We wanted to expose these cells to the normal cardiac milieu in the event that they would undergo a milieu-dependent differentiation and transform into cardiomyocytes.

Dr Robbins. I have two quick follow-up questions. First, do you have any scanning electron microscopy to further delineate the ultrastructure? Second, what do you think about your cells in an ischemic model, such as cryoinjury or ligation of a coronary artery?

Dr Chedrawy. With respect to your first question, scanning electron microscopy was not included as part of the study. However, I agree that it would definitely be of value to clarify the ultrastructure of these cells, especially the cell-to-cell junctions.

With respect to your second question, we have implanted these cells in ischemic models, both cryoinjury and coronary ligation. However, our biggest concern was the actual survival of these cells within the myocardially induced scar.

Dr Robbins. And did they survive?

Dr Chedrawy. As long as 2 weeks.

Dr Richard D. Weisel *(Toronto, Ontario, Canada).* What do you think happens in the ischemic model?

Dr Chedrawy. I think that the biggest concern with the ischemic model is that they are not receiving that stimulus to actually convert into myocardial cells because they are not exposed to the normal environmental cues in the myocardium.

Dr Weisel. To what do they convert?

Dr Chedrawy. Various forms of fibroblasts.

Dr Weisel. So maybe that is not the right answer for infarct replacement.

Dr Joseph B. Shrager *(Philadelphia, Pa).* In terms of showing differentiation toward cardiac muscle, if you are going to use expression of a myosin heavy chain to try to establish this, you have to use something other than generic sarcomeric myosin heavy chain. The α cardiac myosin heavy chain is the only one that is found only in cardiac muscle. Any of the other isoforms of myosin heavy chain are found in skeletal muscle, and therefore their presence does not establish cardiac differentiation.

Dr Chedrawy. We did not look at an α myosin heavy chain. We strictly looked at sarcomeric myosin heavy chain. However, you are right, we could have stained for other cardiac-specific proteins.