Effects of Cellular Cardiomyoplasty on Ventricular Remodeling Assessed by Doppler Echocardiography and Topographic Immunohistochemistry

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**Background** Myocardial infarction (MI) promotes deleterious remodeling of the myocardium, resulting in ventricular dilation and pump dysfunction. Supplementing infarcted myocardium with neonatal myocytes would attenuate deleterious remodeling and so the present study used Doppler echocardiography and histology to analyze the cardiac function and histological regeneration of the damaged myocardium after cellular cardiomyoplasty.

**Methods and Results** Experimental MI was induced by 24-h coronary ligation followed by reperfusion in adult male Lewis rats and neonatal myocytes were injected directly into the infarct and peri-infarct regions. Three groups of animals were studied at 4 weeks after cellular cardiomyoplasty: noninfarcted control (control), MI plus sham injection (MI), and MI plus cell injection (MI + cell). Ventricular remodeling and cardiac performance were assessed by Doppler echocardiography or contrast echocardiography. At 4 weeks after cellular cardiomyoplasty, MI + cell hearts exhibited attenuation of global ventricular dilation and cardiac function compared with MI hearts not receiving cellular cardiomyoplasty. Immunohistochemically, connexin-43-positive small cells were observed in the vicinity of the infarction in MI + cell heart. By electron microscopy, these cells contained myofilaments with Z-bands and poorly developed intercalated disks, suggesting neonatal myocardial cells. Furthermore, the myocardial cells were often making close contact with interstitial cells.

**Conclusions** Implanted neonatal myocytes form viable grafts after MI, resulting in attenuated ventricular dilation and enhanced contractile function. Echocardiography, electron microscopy, and immunohistochemistry are useful methods for assessing the functional and histological regeneration of the damaged myocardium. (Circ J 2004; 68: 580–586)

**Key Words:** Cell therapy; Echocardiography; Myocardial infarction; Remodeling

Despite advances in the treatment of myocardial infarction (MI), congestive heart failure secondary to infarction continues to be a major complication. MI promotes acute and chronic transformation of both the necrotic infarct zone and the nonnecrotic, peri-infarct tissue, leading to global alterations that have collectively been termed ‘ventricular remodeling’.1,2 The cardiomyocytes lost during an MI cannot be regenerated, and the extent of the loss is inversely related to cardiac output, pressure-generating capacity, and, ultimately, survival.3,4 Cellular cardiomyoplasty, or the supplementation of tissue with exogenous cells, has previously been used in the treatment of disease in which terminally differentiated cells are irreparably damaged and supplementing infarcted myocardium with fetal or neonatal myocytes would result in the formation of viable muscle grafts capable of attenuating deleterious post-MI remodeling.5,7

The recent development of cellular cardiomyoplasty offers a new approach to restore impaired heart function8 and evaluation of the survival of the transplanted cells and estimation of their ultimate effect on left ventricular function are very important issues. Doppler echocardiography is a useful tool for measuring cardiac systolic and diastolic function. In this study, we used invasive hemodynamic studies and non-invasive Doppler echocardiography to evaluate the cardiac function of rats with myocardial infarcts after the injection of neonatal cardiomyocytes or culture media alone. In addition, cell survival and cell–cell attachment were analyzed by electron microscopy, and immunohistochemistry.

**Methods**

**Myocardial Infarction Model**

Lewis strain male rats (300 g, 8 weeks old; Seac Yoshitomi Ltd, Fukuoka, Japan) were cared for humanely, in compliance 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 Resource and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985). Acute MI was induced as described elsewhere.
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Cell Isolation and Purification

Rat neonatal ventricular myocytes were isolated as previously reported. In brief, apical halves of the cardiac ventricles from 1- to 2-day-old Lewis rats were separated, minced, and dispersed with 80 U/ml collagenase IV and 0.6 mg/ml pancreatin. A discontinuous gradient of Percoll was used to segregate myocytes from nonmyocytes. After centrifugation for 30 min at room temperature, the upper layer consisted of a mixed population of nonmyocyte cell types, and the lower layer consisted almost exclusively of cardiac myocytes. After the myocytes were incubated on uncoated 10 cm culture dishes for 30 min to remove any remaining nonmyocytes, the nonattached viable cells were used for cell transplantation.

Animal Groups and Cardiomyocyte Transplantation (Cellular Cardiomyoplasty)

Three groups of animals were studied: control animals receiving neither infarction nor implantation and only injected with culture medium (control), infarcted animals not receiving neonatal cardiomyocyte therapy (MI), and infarcted animals receiving neonatal cardiomyocyte therapy (MI + cell). Cells or medium were injected into the infarct area with a 26-gauge needle bent at an angle of 45°, 3 mm from the tip. This configuration was designed to facilitate injection into the infarct. Rats that survived to 24 h after coronary artery occlusion were randomized to receive either injection of cells (3–5 × 10^6/50 μl) or medium (50 μl). Cardiac echocardiography was performed at 24 h after MI and 4 weeks after cellular cardiomyoplasty.

Hemodynamic and Doppler Echocardiographic Studies

Rats were lightly anesthetized with ketamine HCl (25 mg/kg, ip) and xylazine (10 mg/kg, ip). Echocardiography was performed using a commercially available echocardiographic system equipped with a 12-MHz phased-array transducer (SONOS 5500, Phillips, Andover, MA, USA). A 2-dimensional short-axis view of the LV was obtained at the level of the papillary muscles. Ejection fraction was measured by modifying Simpson’s method, which uses 4-chamber views (Fig 1).10 If the left ventricular chamber could not be gained clearly, we used contrast echocardiography. The contrast agent, optison, was injected into the left ventricular cavity via the femoral vein (Fig 2). Pulsed-
wave Doppler spectra of mitral inflow velocities were recorded from the apical 4-chamber view, with the sample volume placed near the tips of the mitral leaflets and adjusted to the position at which velocity was maximal and the flow pattern laminar. Sample volume was set at the smallest size available. All Doppler spectra were recorded on paper speed at 200 mm/s and analyzed off-line. The hemodynamic studies are performed at 4 weeks after MI.

Light and Electron Microscopy Studies

For the light microscopic study, the specimens were fixed in 10% formaldehyde, embedded in paraffin, and cut into 4-μm-thick sections, which were stained with hematoxylin and eosin, and Mallory-azan.

For the electron microscopic study, the specimens were fixed in 4% paraformaldehyde containing 0.25% glutaraldehyde and 4.5% sucrose, postfixed in 1% osmium tetroxide, dehydrated through passage in a series of graded ethanol, and embedded in Epon. Ultrathin sections obtained from the embedded blocks were stained with uranyl acetate and lead citrate, and were examined with a Hitachi H-7000 electron microscope.

Immunohistochemistry

Additional sections were obtained from the paraffin block for the immunohistochemical microscopic study. The sections were placed on silanized glass slides (No.S3003, DAKO Japan, Kyoto, Japan), which were then dried, dewaxed in xylene, and rehydrated in graded concentrations of ethanol. After incubation with normal blocking serum, the sections were incubated overnight at 4°C with monoclonal antibody against connexin-43 (ZYM#71-0700, Zymed Laboratories, Inc, South San Francisco, CA, USA). After incubation with secondary antibody, the sections were allowed to react with Vectastain Elite ABC reagent (Vector Laboratories, Burlingame, CA, USA), then reacted with the peroxidase substrate solution (Vectastain 3'3'-diaminobenzidine substrate kit; Vector Laboratories).

As a positive control for the immunohistochemical procedure, neonatal rat heart fixed in paraformaldehyde was treated in the same way. As a negative control, some sections were first incubated with mouse ascites fluid, which was obtained from animals injected with non-secreting hybridoma cells, and the treated with the second-stage reagent as described.

Statistics

All results are expressed as mean ± SEM. Statistical significance was determined using ANOVA. Differences were considered significant at p<0.05.

Results

Functional Assessment of the Infarcted Myocardium Before and After Cellular Cardiomyoplasty

The left ventricular end-diastolic dimension (LVDd),
fractional shortening (FS) and E/A (E wave = peak early rapid filling wave/A wave = late filling wave because of atrial contraction) at baseline were not significantly different between the 2 groups of MI rats (non-treated and treated groups; LVDd: 7.4±0.2 mm, 7.5±0.3 mm, NS; %FS: 29±3, 30±2, NS; E/A: 3.2±0.9, 3.1±0.8, NS). Left ventricular cavity size significantly increased in rats with MI at 4 weeks and the rats with MI had significant systolic dysfunction, as evidenced by decreased %FS and ejection fraction (EF). Cellular cardiomyoplasty significantly prevented the left ventricular cavity dilatation, decreases in %FS and EF, and increase in E/A at 4 weeks (Fig 3, Table 1).

**Effects of Cellular Cardiomyoplasty on Hemodynamics and Heart Weight (Table 2)**

In treated rats, the left ventricular end-diastolic pressure (LVEDP) decreased (MI: 19±1 mmHg, MI + cell: 11±1 mmHg; p<0.01). Right ventricular weight was significantly higher in rats with MI (0.76±0.03 mg/g) than in controls (0.58±0.02 mg/g). Cellular cardiomyoplasty prevented an increase in right ventricular weight (0.62±0.04 mg/g).

**Light Microscopy and Immunohistochemistry**

In the MI rats that did not receive cellular cardiomyoplasty, replacement fibrosis and interstitial cells were prominent, corresponding to the histological findings of an old MI (Fig 4). In the cellular cardiomyoplasty group, however, the extent of fibrosis tended to be small. Intriguingly, spindle-shaped cells, apparently different from fibrocytes, were often found in the interstitium adjacent to the infarcted area. Immunohistochemically, connexin-43 was only observed in the cytoplasm of cells in the cellular therapy group (Fig 4) and these connexin-43-positive cells were scattered around capillaries preserved in the infarcted area; they were not found in the control group or the untreated MI group.

**Electron Microscopy**

Under electron microscopy, myocardial cells far from the infarcted area had a normal configuration, and fully developed intercalated discs and gap junctions were observed (Fig 5A, B). On the other hand, those in the vicinity of the infarcted area were in various stages of degeneration, including vacuolar formation, destruction of the mitochondrial cristae, partial dissociation of the intercalated discs, and necrosis with contraction bands (Fig 5C, D).

In the cellular therapy group, clusters of small cells (2–3 μm in width and 6–7 μm in length) were observed in the infarcted area, some of which contained myofilaments with Z-bands in the cytoplasm suggesting myocardial cells (Figs 5C, 6). The intercalated discs of the myocardial cells were poorly developed and no gap junctions were observed, which meant such cells were less mature myocardial cells and might be implanted neonatal myocytes. Intriguingly,
Fig 5. Light micrograph (C) and electron micrographs (A, B, and D). Ultrastructure of cardiomyocytes far from the infarcted area showed normal configuration (A), and developed intercalated disc and gap junction were observed (B). Semi-thin (1 μm) section stained with toluidine blue was obtained to make an orientation before the electron microscopy (C). In the cellular therapy group, a cluster of small cells was observed within a circle. One of residual cardiomyocytes after the infarction (arrow) showed a variety of degeneration, including vacuolar formation, disarrangement of myofilaments, and contraction bands (D). Scale bar, 1 μm.

Fig 6. Electron micrographs. In the cellular therapy group, a cluster of small cells was observed in the infarcted area, one of which contained myofilaments with Z-bands (arrows) and poorly developed intercalated discs suggesting neonatal myocardial cell (A). The rectangular part is enlarged (B). The less mature state of myocardial cell is making close contact with an interstitial cell (arrows). Three or 4 cells making close contact each other in the interstitium, one of which might be a myocardial cell as myofilaments are observed in the cytoplasm (asterisk) (C). An interstitial cell has protruded a part of cytoplasm making contact via pseudopodia with a cardiomyocyte (D).
these immature myocardial cells were occasionally making point-to-point contact with interstitial cells (Fig 6).

Discussion

Remodeling of the left ventricle after MI, which is a major cause of infarct-related heart failure and death, depends on acute and chronic transformation of both the necrotic infarct region and the non-necrotic, peri-infarct tissue. Despite pharmacotherapeutics and mechanical interventions, the cardiomyocytes lost during MI cannot be regenerated. The recent finding that a small population of the cardiac muscle cells is able to replicate itself is encouraging, but is still consistent with the concept that such regeneration is restricted to viable myocardium. Over the past several years, increasing awareness of the shortcomings of heart transplantation and left ventricular assist system implantation has led to the consideration of alternative means of treating end-stage heart failure. In this study, we showed by echocardiography that cellular cardiomyoplasty prevented left ventricular remodeling and that transplanted neonatal cardiomyocyte formed viable grafts in infarcted hearts.

As indicated by echocardiography, the rats with MI had significant systolic and diastolic dysfunction, as shown by the significant decrease in %FS, EF and the increase in the E/A ratio. We found that cellular cardiomyoplasty significantly improved cardiac dysfunction in these rats. Doppler echocardiography is the technique for evaluating left ventricular diastolic function6 and increased E wave velocity, decreased peak A wave velocity (or absent A wave), and rapid E wave deceleration were observed in the rats, and these flow patterns were similar to the transmitral flow profiles observed in patients with heart failure with restrictive patterns. Improvement of E/A ratio is caused by preload, afterload reduction, improvement in left ventricular relaxation, or a decrease in passive elastic properties. Our data do not directly answer the question of whether the changes in the E/A ratio are caused by changes in myocardial properties, changes in left ventricle loading conditions, or both. We suppose that the improvements in the E/A ratio are caused by changes in myocardial properties or a decrease in passive elastic properties. Our data do not suggest that cellular cardiomyoplasty prevented left ventricular remodeling and that transplanted neonatal cardiomyocyte formed viable grafts in infarcted hearts.

Ultrastructural observation supported the possibility that the spindle-shaped cells observed in the cellular therapy group were neonatal myocardial cells; that is, clusters of small cells (2–3 μm wide and 6–7 μm long) were observed in the MI+cell group, and some of the small cells contained myofilaments with Z-bands in the cytoplasm suggesting myocardial cells (Figs 5C, 6). Furthermore, the intercalated discs of these cells were poorly developed and no gap junctions were observed, which mean the small cells observed in the MI+cell group were less mature (Fig 5E). Thus newborn myocardial cells implanted on the day following MI might have survived for 4 weeks in the rat heart.

Another important issue is the cell-cell attachment in the cellular therapy group. This is the first report to detect immature myocardial cells making close contact with interstitial cells. Under electron microscopy, some interstitial cells were observed to be protruding part of their cytoplasm to make contact with cardiomyocytes (Fig 6). It might be that transplanted cardiomyocytes need to make close contact with interstitial cells to adjust to the microenvironment and thus survive in the infarcted area. Further examination focusing on the cellular contact in cardiomyoplasty should be done.

We do not have any data to show that the neonatal myocytes survived in the myocardium. In a previous study, 15% of neonatal cardiomyocytes survived for at least 12 weeks when transplanted into healthy rat myocardium. In the infarcted myocardium, based on quantitative TaqMan PCR analysis, approximately 60% of injected neonatal cardiac myocytes would have survived, but the actual number will be lower because >10% of the injected cells were noncardiomyocytes such as fibroblasts or endothelial cells, which may be more resistant to the physical strain of injection and hypoxia and may also have proliferated. Although global contractile function was increased after cellular implantation, the reason remains uncertain and there may be several mechanisms responsible for the improved cardiac function. One is that implanted neonatal cardiomyocyte are actively responsible for force generation during the cardiac cycle. Another is that enhanced cardiac function is more likely to be a result of overall attenuation of deleterious ventricular remodeling within the infarcted and viable myocardium rather than an active force generation by cardiomyocytes. In addition, it is possible that growth factors, released by implanted cells, may exert a protective effect through stimulation of angiogenesis within the infarcted and noninfarct regions. However, the physiology underlying the improved cardiac function remains speculative.

In conclusion, we have demonstrated that neonatal cardiomyocytes existed in the damaged myocardium and prevented the dilatation of the impaired myocardial wall. Doppler echocardiography, electron microscopy, and immunohistochemistry are useful tools for assessing the effect of cellular cardiomyoplasty on the course of MI.

References


