Formation of Cell Junctions Between Grafted and Host Cardiomyocytes at the Border Zone of Rat Myocardial Infarction

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Background—Cardiomyocyte transplantation is an innovative strategy for the treatment of heart failure after myocardial infarction. Cell junctions show diverse temporal polarization toward intercalated disks during postnatal development and exhibit altered distribution in diseased hearts. To elucidate the formation of cell junctions between grafted and host cardiomyocytes at the border zone of myocardial infarction, the 3D distribution of cell junctions was examined using immunohistochemistry and confocal microscopy.

Methods and Results—Neonatal cardiomyocytes obtained from 3-day-old rats by collagenase digestion and Percoll density centrifugation were injected into the border zones of infarction sites 10 days after coronary ligation in adult rats. At 4 to 14 days after transplantation, hearts were harvested and processed by immunohistochemistry. Antibodies against connexin43, desmoplakin, and cadherin were used to analyze the distribution of gap junctions, desmosomes, and adherens junctions, respectively. Grafted cardiomyocytes were identified by immunohistochemistry for α-smooth muscle actin. Grafted cardiomyocytes tended to align parallel to the host cardiomyocytes. Connexin43, desmoplakin, and cadherin were localized between grafted cardiomyocytes themselves and between grafted and host cardiomyocytes. Semiquantitative analysis revealed that all junctions showed increasing polarization to longitudinal cell termini, especially at the border of grafted and host cardiomyocytes, as time advanced from 4 to 7 days after transplantation.

Conclusions—These findings indicate that grafted cardiomyocytes foster electrical pathways with host counterparts through the gap junction and suggest that the environment in infarcted hearts could influence the localization of gap junctions, desmosomes, and adherens junctions. (Circulation. 1999;100[1]:II-262–II-268.)

Key Words: gap junctions ▪ transplantation ▪ intercellular junctions ▪ intercalated disk ▪ myocardial infarction

Altered gap-junction distribution occurs in ischemic heart disease.1–4 The abnormal distribution of gap junctions in the epicardial border zone of infarcts might contribute to arrhythmogenesis.5 In the adult ventricle, gap junctions showed precise 3D patterns of colocalization with desmosomes and adherens junctions at intercalated disks. This pattern of gap junction organization is not present in newborn mammals; it develops throughout postnatal life in association with decreases in side-to-side connections.6–8 This process is vital to the emergence of the uniform anisotropic conduction of action potentials, an electrophysiological characteristic of mature myocardial tissues, and to the rapid and efficient depolarization of cardiac muscles.7

Transplantation of cardiomyocytes into the scarred myocardium has been proposed as a new approach to treat heart failure after myocardial infarction. Some data have shown improved function of infarcted myocardium after cardiomyocyte transplantation.9,10 To improve the function of the diseased heart, it is necessary for grafted cardiomyocytes to perform orderly and synchronous mechanical contractions coincident with the host myocardium. Although several studies showed the formation of intercalated disks between host/grafted or grafted/grafted cardiomyocytes11–14 and overt gap junction structure was demonstrated by Koh et al,11 a detailed observation of the 3 types of cell junctions has not been reported. In the present study, we demonstrated the 3D expression of cell junctions (gap junctions, desmosomes, and adherens junctions) between grafted and host cardiomyocytes and compared them with the distribution of these junctions during postnatal development of the normal heart, junctions of cultured cardiomyocytes, and junctions of cardiomyocytes at the border zone of myocardial infarction. We did this using immunohistochemistry and confocal laser scanning microscopy.

Methods

This study adhered to the standards detailed in Principles of Laboratory Animal Care by the National Institutes of Health (NIH...
Cardiomyocyte Isolation and Culture

Neonatal rat cardiomyocytes were isolated and purified by modifying a previously described method. Briefly, the heart ventricles of 3-day-old Wistar rats (Nikon Doubutsu, Osaka) were separated from the atria and excised under ether anesthesia. The ventricles were washed with PBS containing (in mmol/L): NaCl 136.9, KCl 2.68, Na2HPO4 8.10, KH2PO4 1.47, CaCl2 0.90, and MgCl2·6H2O 0.49 (pH 7.4) and then incubated in PBS without CaCl2 and MgCl2. After this, the ventricles were minced and incubated in 10 mL of PBS containing 0.2% collagenase for 30 minutes at 37°C. The procedures were repeated twice. The cells were then isolated by repetitive pipetting of the digested ventricular tissues. The cells in the supernatant were transferred into a tube containing 20 mL of cell culture medium (DMEM [Gibco] containing 10% fetal bovine serum, 600 μg/mL L-glutamine, 1.9 mg/mL NaHCO3, and 50 μg/mL gentamicin) that was previously filtered through a 30-μm nylon mesh. The tube was centrifuged at 200g for 5 minutes at room temperature, and the cell pellet was resuspended in the cell culture medium for purification. The cardiomyocytes present in the suspension were separated from other cells (ie, fibroblasts and endothelial cells) by the density centrifugation method. The cell suspension was then layered onto 40.5% Percoll (Pharmacia) diluted in HEPES buffer (it had previously been filtered through a 30-μm nylon mesh) that had been previously centrifuged at 200g for 5 minutes at room temperature. Cardiomyocytes were retrieved from the interface of the 40.5% and 58.5% Percoll concentrations. Retrieved cells were then resuspended in the cell culture medium. The suspension, which was diluted to achieve a final concentration of 1×10^6 cells/mL, was plated on a cell-culture dish before it was incubated for 24 hours at 37°C in a CO2 incubator. The cardiomyocytes were further cultured for a 24-hour period in a similar culture medium and used for transplantation.

To compare the distribution patterns of the cell junctions of grafted cardiomyocytes in vivo with those of cultured cardiomyocytes on aligned collagen gels, some cardiomyocytes were cultured on the dishes that had previously been coated with collagen gels. Briefly, 500 μL of 10× minimal essential medium (Gibco) was mixed with 500 μL of 200 mmol/L HEPES buffer (final pH 7.4), and a 3.5-ml layer of collagen type I (VITROGEN 100, 3.0 mg/mL; Collagen) was added for coating. The solution was diluted with 5.5, KCl 5.4, and MgSO4 0.8 [pH 7.35], which had previously been layered on 58.5% Percoll diluted in HEPES buffer. The cell suspension was then centrifuged at 1500g for 30 minutes at room temperature. Cardiomyocytes were retrieved from the interface of the 40.5% and 58.5% Percoll concentrations. Retrieved cells were then resuspended in the cell culture medium. The suspension, which had been previously centrifuged at 200g for 5 minutes at 37°C in a CO2 incubator. The cardiomyocytes were further cultured for a 24-hour period in a similar culture medium and used for transplantation.

Cardiomyocyte Transplantation

Cultured cardiomyocytes were washed with PBS to remove dead cells. After detachment from the culture dish with dispase (Gou-doushusei), cells were collected in a tube and centrifuged at 200g for 5 minutes at room temperature. The cell pellet was resuspended in the culture medium at 1×10^6 cells/50 μL.

Cardiomyocyte transplantation was performed 10 days after myocardial infarction. The rats were anesthetized and ventilated as described above, and the hearts were exposed through a left thoracotomy. The cardiomyocyte suspension (50 μL) was injected into the border zone of the predicted infarction using a 27-G needle and a tuberculin syringe. The injection site was marked with a small suture (7-0 polypropylene). Sham-operated rats (n=2) were injected with 50 μL of culture medium alone at the border zone of the infarction site. After the chest incisions were closed in layers, the rats were allowed to recover in their cages.

Tissue Preparation

Hearts were excised under ether anesthesia after cardiomyocyte transplantation at various times (days 4, 7, and 14) and were fixed by perfusion of 2% paraformaldehyde with 0.01 mol/L PBS via the coronary arteries for 5 minutes. Then, hearts were transversely sectioned across the infarct into 2 blocks, and the tissue blocks were immersion-fixed in 2% paraformaldehyde with 0.01 mol/L PBS at 4°C for 2 hours. After gradual infiltration with Tissue-Tek OCT compound (Miles), the specimens were frozen in liquid nitrogen or dry-ice/acetone and stored at −70°C until use.

Antibodies

For immunohistochemical examination, antibodies against connexin43 (Cx43), desmoplakin, and pan-cadherin, which are the respective major constituents of the cardiac gap junction, desmosomal plaque, and the adherens junction, were used to detect cell junctions. The following antibodies were used: rabbit polyclonal antibody against a Cx43-specific peptide (Zymed Laboratories), mouse monoclonal antibody against desmoplakin I+II (Boehringer Mannheim), and mouse monoclonal antibody against pan-cadherin (Chemicon). For detection of primary antibodies, we used Texas Red-labeled anti-rabbit IgG (Vector Laboratories) and Texas Red-labeled anti-mouse IgG (Vector Laboratories). To identify the presence of neonatal graft cells, FITC-labeled monoclonal antibody against α-smooth muscle actin isoform (αSMA) (Sigma), which is present in fetal cardiomyocytes but not in adult cardiomyocytes, was used. The above-mentioned antibodies against mouse IgG were absorbed with normal rat serum.
Immunofluorescence

Serial 20-μm-thick frozen sections were prepared with a cryostat microtome (Bright 5030 microtome) and placed on silane-coated slides (Matsunami). Sections were stored at −70°C until use. After thawing to room temperature, sections were rinsed in PBS, fixed with ethanol at −20°C for 10 minutes, and dried. The sections were then rinsed in PBS containing 5% skim milk for 15 minutes and washed in PBS containing 0.1% Triton X-100 for 15 minutes. Double immunolabeling was performed using antibodies and reagents in the following sequence: rabbit anti-Cx43 antibody (1:500 dilution) was used overnight at 4°C, and then a mixture of Texas Red-labeled anti-rabbit IgG (1:200) and FITC-labeled anti-αSMA (1:300) was used for 2 hours at 37°C. When mouse anti-desmoplakin antibody or mouse anti–pan-cadherin antibodies were used, the following procedure for staining the same specimen with the 2 mouse monoclonal antibodies was used. (1) Specimens were incubated with either mouse anti-desmoplakin antibody (1:20) or mouse anti–pan-cadherin antibody (1:500) overnight at 4°C; (2) Texas Red-labeled anti-rabbit IgG (1:200) was used for 2 hours at 37°C; (3) mouse monoclonal antibody was used for 1 hour at 37°C; (4) goat anti-mouse IgG Fab fragment (1:20) was used for 1 hour at 37°C; and (5) FITC-labeled anti-αSMA (1:300) was used for 2 hours at 37°C. Finally, specimens were rinsed with PBS and mounted in Vectashield (Vector) before being examined with confocal laser scanning microscopy (FluoView, Olympus). Digital images were transferred to a Macintosh computer (Apple) equipped with Photoshop software (Adobe) and then printed (PM-750C, Epson).

Semiquantitative Analysis of Cell Junction Area

To assess myocardial cell junctions, all aspects of tissue processing, labeling, and image analysis were standardized. The pixels of fluorescently labeled areas for each protein at the cell boundaries in grafted and host cardiomyocytes on aligned collagen gels were compared. Confocal images were collected using an objective lens (×60) with a 1-time zooming (field, 53000 μm²). The “black level” was constant, such that the outlines of individual cardiomyocytes were visible, and the “gain” control was adjusted so that the spectrum of label intensities spanned the full 255-level scale. The digital images that were transferred to the Macintosh computer were analyzed with image-analysis software (NIH Image).

Fields of grafted and host cardiomyocytes, which were stained with and without FITC-labeled anti-αSMA, respectively, were measured by enclosing the cell boundaries by hand. This enclosure was edited by hand to remove all extraneous lipofuscin and blood vessel autofluorescence. Fluorescent areas of each cell-junction type were thus measured. A binary overlay was created automatically by a set threshold of 50 on the 255-point gray scale to eliminate the background cell outlines; in this onlay, each pixel was either on or off. The areas at the longitudinal cell termini were analyzed in each field by enclosing the field by hand before measuring the binary overlaid area in it.

To minimize errors introduced by variations among immunohistochemical labeling runs, samples of each group were included in every run. The mean areas of 3 sampling sites from the positive control tissues were used to ensure consistency of the technique among labeling runs.

Results

Left Ventricles After Myocardial Infarction

One to 3 weeks after myocardial infarction, the longitudinal edges of cardiomyocytes facing the infarct extended cytoplasmic cell processes, with clear striation of F-actin toward the infarct. Immunolabeling of Cx43, desmoplakin, and cadherin was distributed between the cytoplasmic processes, which fostered intracellular and intercellular junctions. Immunolabeling of the 3 cell junctions also appeared at the transverse cell termi of cardiomyocytes at the border zone of myocardial infarction (Figure 1).

Grafted Cardiomyocytes in Myocardial Infarction

Of the 18 transplanted rats, 2 died unexpectedly by 2 days after transplantation.

On days 4 and 7 after transplantation, fusiform-grafted cardiomyocytes with clear striations were identified at the border zone of myocardial infarction using αSMA immunostaining (Figures 2 and 3). Examination of control sections taken from the sham-operated hearts revealed that only smooth muscle cells of the vascular wall were positively stained with the anti-αSMA antibody. Most surviving grafted cardiomyocytes were aligned parallel with the host myocardium. The longitudinal cell borders of grafted cardiomyocytes facing other cardiomyocytes (in-
including grafted or host cardiomyocytes) were arranged in a cylinder-like structure. However, the longitudinal cell borders of grafted cardiomyocytes facing the infarcted area were structured into complex cell processes similar to the host cardiomyocytes facing the infarct. We could not further identify the grafted cardiomyocytes by 14 days after transplantation.

Cx43, desmoplakin, and cadherin were clearly expressed at the cell borders of the grafted/grafted and host/grafted cardiomyocytes by 4 days after transplantation (Figures 2 and 3). Of these junctional proteins, the fluorescent dot densities of desmoplakin and cadherin surpassed those of Cx43. Junctional proteins were predominantly distributed at the longitudinal cell termini 7 days after transplantation (Figure 3, a and b); even the grafted cardiomyocytes were surrounded by host cardiomyocytes (Figure 3c). In addition, the immunolabeled spots between the cell processes of grafted cardiomyocytes facing the infarct were similar to those of host cardiomyocytes facing the infarct (Figure 3b).

Normal Left Myocardium in Postnatal Development
Sequential confocal images taken from 20-μm-thick sections revealed clear 3D distribution of Cx43, desmoplakin, and cadherin as aggregates of fluorescent domains at intercalated disks between cardiomyocytes in 90-day-old rats. At the transverse cell edges, some fluorescent spots of these cell junctions exhibited mutual expression. However, immunolabeling of the 3 junctional proteins appeared in relatively dispersed patterns on the cell membranes of 1-day-old rats. During postnatal development, preferential localization of desmoplakin and cadherin at longitudinal cell termini occurred earlier than that of Cx43.

Cultured Cardiomyocytes on Aligned Collagen Gels
Cultured cardiomyocytes with a fusiform shape dispersed in a fashion parallel to the axis of collagen gels, and they acquired synchronous contractile activities after 48 hours of

Figure 3. Confocal images of rat left ventricle labeled for αSMA (SMA) and for Cx43 (Cx) (a) and desmoplakin (DP) (b) and a stereoimage of αSMA and Cx43 (c) 7 days after transplantation. Infarct zones are shown by * (a and b). Cardiomyocytes were aligned mainly in sites parallel to arrangement of host cardiomyocytes at border zone of myocardial infarction. Cx43 and desmoplakin were localized in grafted cardiomyocytes mainly at longitudinal cell termini in grafted/grafted, host/grafted (red arrowheads), and host/host cardiomyocytes. Note that longitudinal edges of grafted cardiomyocytes facing scarred tissues formed cytoplasmic process-like structures (green arrow), with scattered junctional immunolabeling observed in the processes. Bars=20 μm.
culture. This alignment was fully evident at 72 hours post-culture. Cardiomyocytes displayed a rod-like shape and were arrayed parallel to a common axis. Desmoplakin and cadherin immunolabeling of cardiomyocytes on aligned collagen gels was mainly localized at longitudinal cell termini at 72 hours to 7 days postculture (Figure 4a). Cx43 was localized at the longitudinal cell termini and at the transverse cell termini (Figure 4b). However, cardiomyocytes were randomly oriented when they were cultured on nonaligned collagen, and cell junctions were randomly localized around the cardiomyocytes (Figure 4c).

Semiquantitative Analysis
All 3 junctional proteins showed increased polarization to longitudinal cell termini, especially at the grafted/host cardiomyocyte border, as time progressed from 4 to 7 days after transplantation (Figure 5). Desmoplakin and cadherin were preferentially localized at longitudinal cell termini compared with Cx43. When we compared grafted cardiomyocytes 7 days after transplantation with normal hearts at normal developmental stages (Figure 6), the cell junctions in grafted cardiomyocytes were organized at the longitudinal cell termini earlier than the development of intercalated disks in normal hearts from animals of equivalent ages.

Discussion
Cardiomyocyte transplantation to scarred tissues after myocardial infarction improves host myocardial function. The grafted cardiomyocytes derived from a differentiated tumor line survive and proliferate well in vivo. Although grafted fetal cardiac tissues can proliferate and differentiate into mature cardiomyocytes, these cells maintain their fetal
phenotypes, as shown by positive staining for αSMA (>2 months after transplantation. Li et al demonstrated that transplantation is more successful with 18-days of gestation (fetal) cardiomyocytes (92%) than 5-day-old (neonatal) cardiomyocytes (50%), whereas cardiomyocytes isolated from 22-day-old (young) and 32-day-old (adult) rat hearts did not survive in the host heart. In this study, the grafted 3-day-old neonatal rat cardiomyocytes demonstrated, in part, clear positive staining for αSMA by 7 days after transplantation before losing the response by 14 days after transplantation. Although grafted cardiomyocytes might be killed by transplant rejection, fetal allogenic cardiomyocytes have been detected 65 days after transplantation, without the use of immunosuppressive therapy. These findings suggest that these grafted neonatal cardiomyocytes have higher potentials for differentiation into mature cells than grafted fetal cardiomyocytes.

One of the interesting phenomena found in the present study is that the direction of grafted cardiomyocytes was parallel to that of the host cardiomyocytes. According to Cleutjens et al, collagen types I and III are abundantly produced by myofibroblasts at the border zone 10 days after myocardial infarction. We showed, in the present study, that neonatal cardiomyocytes constructed regular end-to-end connections on aligned collagen gels in culture. These findings support the theory that interactions between the extracellular matrix and the cardiomyocytes, as regulated by α1/β1 integrin, are important in determining the phenotype and function of the cardiomyocytes.

In the present study, 4 days after transplantation, gap junctions were distinctly observed between grafted/host cardiomyocytes, and these gap junctions were localized at the longitudinal and transverse termini. By 7 days after transplantation, the localization of gap junctions to longitudinal cell termini in grafted cardiomyocytes was evident. The pattern of gap junctions, desmosomes, and adherens junctions at the longitudinal termini of grafted cardiomyocytes was similar to that of cultured cardiomyocytes on aligned collagen gels. The percentages of the cell junctions at longitudinal termini 4 and 7 days after transplantation corresponded to those of ~15-day-old and 50-day-old hearts in normal development, respectively. The percentages of these cell junctions at longitudinal termini 7 days after transplantation were slightly less than those of the surviving host cardiomyocytes (whose age was ~80 days old). These results suggest that the environment in infarcted hearts, where grafted neonatal cardiomyocytes face high pressure and rich collagen fibrils, could influence the localization of cell junctions.

Several studies correlating the assembly of gap junctions, desmosomes, and adherens junctions in the heart have suggested that during the postnatal development of ventricular myocardium and hypertrophic cardiomyopathy, the association between gap junctions and adherens junctions might play an important role in the maturation of the electromechanical function. At intercalated disks, well-developed desmosomes and adherens junctions provide sites for sarcolemma stabilization, and they are potentially favorable sites for the preservation of gap junctions located either close to or within the body of the disk. However, gap junctions at transverse sides might be so vulnerable to the shearing forces generated by the contraction of neighboring cardiomyocytes that they are selectively internalized or degraded. Understanding the relationships between electrical and mechanical junctions in grafted cardiomyocytes may provide insights into the pathogenesis of cardiac diseases, including abnormalities of gap junction distribution at infarct border zones, and cardiomyopathy. As such, studies on the implications and correlations of aberrant localizations of adherens junctions and desmosomes, with altered gap junction distribution at the border zone of myocardial infarction, are now in progress at our laboratory.

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