Remodeling of Cell-Cell and Cell–Extracellular Matrix Interactions at the Border Zone of Rat Myocardial Infarcts

Tsutomu Matsushita, Masahito Oyamada, Kazushi Fujimoto, Yuko Yasuda, Shinsuke Masuda, Yukio Wada, Takahiro Oka, Tetsuro Takamatsu

Abstract—At the border zone of myocardial infarcts, surviving cardiomyocytes achieve drastic remodeling of cell-cell and cell–extracellular matrix interactions. Spatiotemporal changes in these interactions are likely related to each other and possibly have significant impact on cardiac function. To elucidate the changes, we conducted experimental infarction in rats and performed 3-dimensional analysis of the localization of gap junctions (connexin43), desmosomes (desmoplakin), adherens junctions (cadherin), and integrins (β1-integrin) by immunoconfocal microscopy. After myocardial infarction, changes in the distribution of gap junctions, desmosomes, and adherens junctions showed a similar but nonidentical tendency. In the early phase, gap junctions almost disappeared at stumps (longitudinal edges of cardiomyocytes facing the infarct), and, although desmosomes and adherens junctions decreased, they still remained. In the healing phase, at stumps, connexin43, desmoplakin, and cadherin were closely associated between multiple cell processes originating from a single cardiomyocyte. Electron microscopy confirmed the presence of junctional complexes between the cell processes. β1-Integrin at the cell process increased during the formation of papillary myotendinous junction–like structures. Abnormal localization of connexin43 was often accompanied by desmoplakin and cadherin on lateral surfaces of surviving cardiomyocytes. These findings suggested that remodeling of gap junction distribution was closely linked to changes in desmosomes and adherens junctions and that temporary formation of intracellular junctional complexes was an element of the remodeling of cell-cell and cell–extracellular matrix interactions after myocardial infarction. Moreover, the remodeling of the intercalated disk region at the myocardial interface with area of scar tissues was associated with the acquisition of extracellular matrix and β1-integrin. (Circ Res. 1999;85:1046-1055.)

Key Words: cell junction ■ intercalated disk ■ myocardial infarction ■ remodeling ■ extracellular matrix

Adult mammalian cardiomyocytes have only a limited regenerative potential. Once myocardial infarction occurs, infarcted myocardium undergoes a progressive sequence of changes consisting of ischemic coagulative necrosis, followed by inflammation and replacement with scar tissues. During the sequence, surviving cardiomyocytes have to adapt to an unfavorable environment, including ischemia, and inadequate adaptation results in ventricular remodeling. At the border zone of myocardial infarcts, viable cardiomyocytes undergo drastic reorganization of cell-cell and cell–extracellular matrix (ECM) interactions as they lose cell-cell connections because of the death of neighboring cardiomyocytes and subsequently anchor to scar tissues.

The intercalated disk is a unique structure of cardiomyocytes characterized by a grouping of 3 specialized cell-cell junctions; gap junctions, desmosomes, and adherens junctions.1 Gap junctions facilitate conduction of the cardiac impulse so that the whole heart behaves electrically as a syncytium, whereas desmosomes and adherens junctions ensure transmission of the traction generated by the individual cells throughout the myocardium.

In the ischemic heart, several studies have shown the remodeling of gap junctions by studying for connexin43 (Cx43), a major cardiac gap junction protein.2–5 We have reported the formation of cell processes in cardiomyocytes facing the infarct and the expression of Cx43 in these processes.5 The abnormality in gap junction distribution has been suggested to be one factor in arrhythmogenesis after myocardial infarction.6 Although gap junctions are closely associated with desmosomes and adherens junctions in the normal heart,7,8 few studies have been performed on spatiotemporal changes in desmosomes and adherens junctions after myocardial infarction, which potentially bear importance for the remodeling of gap junctions.2–4,9

Apart from intercellular junctions, ECM plays a vital role in force transmission throughout the myocardium.10,11 ECM
**Figure 1.** Immunoconfocal images of junctional proteins in sham-operated rat left ventricles (A and B) and left ventricles at 6 hours (C and D) and 48 hours (E through G) postligation. *Infarcts on the right (C through G). A, Double-labeling for Cx43 (FITC, Cx) and desmoplakin (TR, DP). Yellow signals indicate very close distribution of both proteins. B, Double-labeling for β1-integrin (FITC, β1) and laminin (TR, La) in left ventricular papillary MTJ. † indicates tendineae connective tissue; ‡, vessel wall. C and D, Double-labeling for either Cx43 (FITC) (C) or desmoplakin (FITC) (D) and actin (TR, phalloidin [Ph]). Desmoplakin signals of infarcted cardiomyocytes are agglomerated and are stronger than Cx43 signals. E and F, Double-labeling for Cx43 (FITC) and either desmoplakin (TR) (E) or cadherin (TR, Cad) (F). Cx43 signals nearly disappear at the stump. G, Double-labeling for β1-integrin (FITC) and laminin (TR). β1-Integrin signals are weakly expressed at the stump (arrowhead). All figures are of the same magnification. Bar=20 μm.
significantly contributes to heart development and cardiac function through integrin-mediated cell-ECM interactions. After myocardial infarction, surviving cardiomyocytes facing the infarct lose their neighboring cells with which intercalated disks have originally formed, leaving blunt-ended stumps in direct contact with ECM.\textsuperscript{12,13} 1-Integrin is very likely to play an important role in this interaction, because it is the predominant \( \beta \)-subunit isoform of adult myocardial integrins\textsuperscript{11,14,15} that are involved in forming cell-surface receptors for various ECM components.

We have postulated the following hypotheses about the remodeling of cell-cell and cell-ECM interactions after myocardial infarction. Firstly, the remodeling of gap junctions after myocardial infarction is linked to changes in desmosomes and adherens junctions. Secondly, remodeling of the intercalated disk region at the myocardial interface with scar tissues requires acquisition of ECM components and integrins. To examine these hypotheses, we analyzed in detail spatiotemporal changes of gap junctions, desmosomes, adherens junctions, and integrins in cardiomyocytes by immunofluorescence microscopy and electron microscopy at the border zone of myocardial infarcts in rats.

**Materials and Methods**

Myocardial infarction was induced in 98 male Wistar rats (9 weeks old) by ligation of a branch of the left coronary artery as previously described.\textsuperscript{5} In the sham-operated group, the ligature was not tightened. Hearts after coronary ligation at various times (6, 12, 24, and 48 hours, and days 3, 4, 8, 15, 30, and 60 postligation) were fixed by perfusion and subsequent immersion with 2% paraformaldehyde in 0.01 mol/L PBS and frozen in liquid nitrogen. For immunohistochemistry of \( \beta \)-integrin, hearts were frozen without fixation.

The following antibodies were used: rabbit polyclonal antibody to Cx43 (Zymed Laboratories), mouse monoclonal antibody (mAb) to Cx43 (Zymed Laboratories), mouse monoclonal antibody (mAb) to cadherin (BD Biosciences), rabbit polyclonal antibody to desmoplakin (BD Biosciences), rabbit polyclonal antibody to \( \beta \)-integrin (BD Biosciences), and mouse monoclonal antibody (mAb) to actin (Cytoskeleton).
Cx43 (Chemicon), mouse mAb to desmoplakin I+II (Boehringer Mannheim), mouse mAb to pancadherin (Chemicon), rabbit polyclonal antibody to laminin (ICN Biomedicals), and hamster mAb to β1-integrin.16 The antibodies against mouse IgG were adsorbed against normal rat serum. Texas Red (TR)-X–labeled phalloidin (Molecular Probes) was used for detection of F-actin. Serially sliced 20-µm-thick frozen sections were fixed with ethanol or acetone, dried, and rinsed in 5% skim milk in PBS, before washing in 0.1% Triton X-100 in PBS (15 minutes). Double-immunolabeling was performed using 2 of the above primary antibodies and FITC- and TR-labeled secondary antibodies. Negative controls in which one of the primary antibodies was excluded were performed to rule out cross-reactivity.

Immunolabeled sections were observed with a confocal laser scanning microscope (FluoView, Olympus). All figures were transverse optical sections of the left myocardium in which the cardio-myocytes were longitudinally sectioned. In the following, we use the terms “borderline” and “vicinity” to discriminate cells located differently with reference to the infarct as previously described.6

For morphometry, each confocal image was collected using an oil-immersion objective lens (PlanApo ×60, numerical aperture=1.4, Olympus) and one-time zooming (field, 265×200 µm²). We classified longitudinal cell boundaries into 3 categories according to their sites with reference to the infarct, ie, those between cells or sites in each of the 3 following categories: infarcted cardiomyocytes (I), stumps (S), and vicinity cardiomyocytes (V). Each longitudinal cell boundary was enclosed manually, and the immunopositive area in the enclosed field and cell width at each cell-cell and cell-matrix boundary were measured. Because the optical section thickness is fixed and intercalated disks were randomly cross-sectioned in our samples, the mean immunopositive area/cell width reflects the amount of junctional proteins per unit area of en face–viewed intercalated disks.17,18 All data were converted so that the mean of each datum on Cx43, desmoplakin, and cadherin of the sham-ligated left ventricle. Double-labeling using anti-Cx43 and either anti-desmoplakin or cadherin antibodies showed that Cx43, desmoplakin, and cadherin were closely distributed with each other in the cell process, and the signal for Cx43 was weaker than those at 48 hours. However, the distribution of these proteins was scattered compared with shams at 48 hours. Cx43 signal was very low at stumps.

Days 3 to 90 Postligation
On day 3 postligation, the localization of desmoplakin and cadherin at stumps approximated that at 48 hours. However, the distribution of these proteins was scattered compared with shams at 48 hours. Cx43 signal was very low at stumps.

On day 4 postligation, TR-phalloidin staining demonstrated that the stumps began to extend fine cytoplasmic cell processes toward the infarct (Figure 2A and 2B). Small immunofluorescent spots of Cx43, desmoplakin, and cadherin were detected in the cell processes (Figure 2A and 2B). Double-labeling for Cx43 and either desmoplakin or cadherin showed that these proteins were closely distributed with each other in the cell process, and the signal for Cx43 was weaker than that of desmoplakin and cadherin (Figure 2C). Small plaques of all 3 junctional proteins were abundant and closely associated on lateral surfaces between borderline and/or vicinity cardiomyocytes. Small plaques gradually decreased with distance from the infarct. The laminin sheath became less apparent in the infarct, and at stumps, β1-integrin was weak at stumps.

Results
Confocal Immunohistochemistry
Left Ventricles and Papillary Muscles of Sham-Operated Rats
Double-labeling using anti-Cx43 and either anti-desmoplakin or anti-cadherin antibodies showed that Cx43, desmoplakin (Figure 1A), and cadherin were mainly closely localized at longitudinal termini of cardiomyocytes in sham-operated left ventricle. Double-labeling using anti-β1-integrin and anti-laminin demonstrated that β1-integrin was localized just inside the laminin sheath around cardiomyocytes in a dotted pattern. Neither β1-integrin nor laminin was observed at the intercalated disk.

Cardiomyocytes terminate on collagenous connective tissue in the chordae tendineae at papillary MTJs. No immunofluorescent dots of these junctional proteins were observed on the MTJ where spindle-shaped cardiomyocytes were facing connective tissues (data not shown). β1-Integrin was densely localized at the MTJ with laminin-forming sheaths around the cardiomyocytes (Figure 1B).

Postligation 6 to 48 Hours
By 6 to 12 hours postligation (Figure 1C and 1D), although signals for Cx43, desmoplakin, and cadherin at the intercalated disks were observed in the infarct zone, their normal distribution was lost, ie, scattered and/or agglomerated. Among the 3 junctional proteins, the number of Cx43 signals significantly decreased compared with those of other proteins in the infarct. At stumps and cell borders of vicinity cardiomyocytes, the expression of Cx43, desmoplakin, and cadherin moderately decreased.

By 24 to 48 hours postligation, the expression of Cx43 markedly decreased to <5% of the levels of sham-operated hearts, whereas those of desmoplakin and cadherin remained at a similar level observed at 6 hours postligation at stumps (Figure 1E and 1F). In the infarct, expressions of Cx43, desmoplakin, and cadherin were greatly diminished, whereas those of vicinity cardiomyocytes decreased moderately. The laminin sheath became discontinuous in the infarct, and at stumps, β1-integrin was weakly expressed accompanied by a small amount of laminin (Figure 1G).

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On days 8 and 15 postligation, borderline cardiomyocytes displayed elongated and intertwined cell processes (Figure 3), portraying clear striations even near the tip. Serial confocal imaging demonstrated that immunofluorescent spots of Cx43 (Figure 3I through 3L), desmoplakin (Figure 3A through 3F), and cadherin were present between the cell processes originating not only from neighboring cardiomyocytes but also from the same cardiomyocyte. The expression of Cx43 was comparable with those of desmoplakin and cadherin at stumps and at cell borders of vicinity cardiomyocytes. Localization of the 3 proteins on lateral surfaces was still observed between borderline and/or vicinity cardiomyocytes (Figure 3H).
3G and 3H). β₁-integrin was expressed on the surface of cell processes. During days 30 to 90 postligation, the number of cell processes at stumps gradually decreased and stumps tapered off. The expression of Cx43, desmoplakin, and cadherin was attenuated (Figure 4A). In contrast, the expression of β₁-integrin increased, especially at the tip of cell processes (Figure 4B).

Morphometry

Figure 5 shows that desmoplakin and cadherin underwent similar changes at all time points. Concerning vicinity cardiomyocytes, the 3 junctional proteins showed similar changes, although the amounts were reduced to ~45% to 60% of those of sham-operated hearts by 48 hours. The protein expression recovered to levels similar to those of sham on days 8 and 15 and decreased on day 60. Concerning the stump, the increase in Cx43 after myocardial infarction was significantly delayed compared with desmoplakin and cadherin on day 4. The expression of the 3 junctional proteins increased to levels similar to those of vicinity cardiomyocytes on days 8 and 15. Furthermore, expression of the 3 proteins at stumps and between vicinity cardiomyocytes decreased on day 60. The time-dependent increase in β₁-integrin at the stump was confirmed by morphometry, and the expression of β₁-integrin gradually approached that of papillary MTJ.

Electron Microscopy

In sham-operated left ventricle, typical intercalated disks existed between cardiomyocytes (data not shown). At 48 hours postligation (Figure 6A), no apparent gap junctional

Figure 4. Double-labeling for Cx43 (FITC) and desmoplakin (TR) on day 60 (A), and β₁-integrin (FITC) and actin (TR, phalloidin) on day 90 postligation (B). Infarct zones on the right. A, Cx43 and desmoplakin signals at stumps are attenuated compared with those on days 8 and 15. Bar=40 μm. B, β₁-Integrin signals are strongly expressed at the stumps. Bar=20 μm. Abbreviations as in Figure 1 legend.

Figure 5. Morphometry for the expression of Cx43 (A), desmoplakin (B), cadherin (C), and β₁-integrin (D) at longitudinal cell termini classified into the following categories: boundaries between infarcted cardiomyocytes (hatched bars), at stumps (solid bars), and of vicinity cardiomyocytes (stippled bars). Data are mean±SD of percentage of immuno-positive areas per cell width compared with sham-operated cardiomyocytes (A through C, open bars). Concerning β₁-integrin, data were expressed as the mean±SD of percentage compared with left ventricular papillary MTJs (PM; open bars). After day 4, data on category I were not obtained, because cardiomyocytes were no longer observed in the infarcted zones. Data were analyzed by 1-factor factorial ANOVA, and significant differences between sham and the categories at all time points were defined by *P<0.05 using the Scheffé test. Concerning β₁-integrin, data were analyzed between the neighboring time points. h, hours; D, day.

Figure 3. Immunoconfocal images of rat left ventricles on days 8 (A through F) and 15 (G through L) postligation. *Infarct zones on the right. Series of confocal optical sections of either desmoplakin (FITC) (A through F) or Cx43 (FITC) (I through L) and actin (TR, phalloidin) taken at 1-μm intervals at stumps. A through F, Desmoplakin signals are distributed between the cell processes originating from mainly the same cardiomyocyte and from neighboring cardiomyocytes (arrowhead in C). Bar= 20 μm. G and H, Double-labeling for Cx43 (FITC) with either actin (TR, phalloidin) (G) or desmoplakin (TR) (H). Specimens are obtained from serial sections. Distribution of Cx43 and desmoplakin becomes more disorganized on approaching the infarct. Cx43 and desmoplakin are closely located at transverse cell boundaries (arrowheads). Bar=40 μm. I through L, Cx43 signals are distributed between the cell processes. Bar=20 μm. Abbreviations as in Figure 1 legend.
Figure 6.
structure was observed between borderline cardiomyocytes and necrotic cardiomyocytes with few remaining myofibrils and cell organelles, although desmosomes and adherens junctions persisted. On day 3 postligation (Figure 6B), cell processes were formed at the stumps, and desmosomes, adherens junctions, and gap junctions were detected between the processes. Basement membranes were occasionally seen around the cell processes. On day 4 postligation (Figure 6C), the stump consisted of multiple small, complex cell processes. Desmosomes and adherens junctions were present between these cell processes, even near the tip. A few subsarcolemmal densities existed at the tip of cell processes surrounded by basement membranes and collagen fibers. On days 8 and 15 postligation (Figure 6D through 6F), intercalated disk-like junctional complexes containing gap junctions, desmosomes, and adherens junctions existed in a side-by-side and an end-to-end manner between elongated cell processes, which had originated from a single cardiomyocyte. The surfaces of cell processes were surrounded by basement membranes, and subsarcolemmal densities were abundantly present at the tip of cell processes. A few contacts between fibroblasts and cardiomyocytes were observed, but no apparent gap junctions, desmosomes, and adherens junctions were recognizable between them (data not shown). On day 60 postligation (Figure 6G), well-developed subsarcolemmal densities were detected at the tip of cell processes, distributed in a pattern similar to that of MTJs of papillary muscle. However, intercalated disk-like structures were barely detected near the tip. We found no significant differences in the localization of gap junctions, desmosomes, adherens junctions and subsarcolemmal densities between non-arrested and arrested hearts after myocardial infarction (eg, compare Figure 6D with Figure 6E).

Discussion
We have shown here that remodeling of gap junctions after myocardial infarction is closely related to changes in desmosomes and adherens junctions and that remodeling of the intercalated disk region at the myocardial interface with areas of scar tissue is associated with acquisition of laminin and $\beta_1$-integrin.

We revealed that abnormal localization of gap junctions on the lateral surface between borderline and/or vicinity cardiomyocytes after myocardial infarction often corresponded to that of desmosomes and adherens junctions, consistent with ultrastructural results in infarct zone cardiomyocytes previously reported. Our present results showing that abnormal distribution of cell junctions occurred at both borderline cardiomyocytes (in contact with scar tissues) and vicinity cardiomyocytes (not in contact with scar tissues), suggest that direct contact with the scar tissue is not essential to cause the abnormality, but rather, indirect effects such as ischemia may cause the abnormality. The impact of chronic ischemia on the expression of the junctional proteins was also indicated by our data that show that signals for Cx43, desmoplakin, and cadherin decreased on day 60.

The association of gap junctions with adherens junctions may be attributed to regulation of gap junctions by cadherins, a mechanism reported not only in epithelial cells but also in cardiomyocytes. In addition, we presume that differences in vulnerability to ischemia exist between gap junctions and cell adhesion junctions, because gap junctions attenuate more rapidly than desmosomes and adherens junctions at the early phase. One example of the divergence among the 3 junctions has been reported during postnatal differentiation of ventricular myocardium. The vulnerability of gap junctions may be attributed to the very rapid turnover of connexins and to the lack of their interaction with the cytoskeleton (desmin and actin) to which desmosomes and adherens junctions are bound.

Although the presence of intracellular junctions has previously been reported in cardiomyocytes in human and canine diseased hearts by electron microscopy, it is difficult to convincingly demonstrate intracellular junctions in ultrathin sections of heart tissues. Our serial confocal imaging clearly demonstrated that contacts were formed between cell processes from neighboring cardiomyocytes (intercellular) but also from the same cardiomyocyte (intracellular). Cx43, desmoplakin, and cadherin were present between them. By electron microscopy, we frequently encountered junctional complexes containing gap junctions, desmosomes, and adherens junctions between intricately intertwined cell processes of the same cell, whereas annular gap junctions in the cytoplasm were rarely observed.

The expression of $\beta_1$-integrin and the extent of subsarcolemmal densities by electron microscopy corresponded well with the formation of multiple elongated cell processes at stumps and subsequent anchoring of cardiomyocytes to scar tissues. The cardiomyocyte attachment to scar tissues resembles papillary MTJs with respect to the amounts of integrins and subsarcolemmal densities.

Figure 7 summarizes the remodeling process of cell-cell and cell-ECM interactions at the stump during myocardial infarction. At early phase postligation (48 hours), borderline cardiomyocytes facing the infarct lose neighboring cells and form blunt-ended stumps while maintaining some desmo-
abnormal conduction in the healing infarct border-zone myocardium, as previously indicated. To the functional significance of the intracellular gap junctions is unclear; this may contribute to arrhythmias such as aberrant distribution of gap junctions on the lateral surfaces, or, alternatively, they function as antiarhythmic structures (canceling out harmful electrical impulses by providing a pathway for the collision of impulses). Intracellular adhesion junctions may coordinate the cytoskeleton in forming attachments of cardiomyocytes to scar tissues in concert with integrins. Alternatively, given that mutations in cytoskeletal protein genes, such as dystrophin and metavinculin, have been found in inherited dilated cardiomyopathy, irregular distribution of intracellular junctions may contribute to a disruption of the cytoskeleton and onset of dilated myopathy.

Further investigations are needed to elucidate molecular mechanisms for the remodeling of cell-cell and cell-ECM interaction during myocardial infarction, which could lead to treatment and/or prevention of complications after myocardial infarction.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from The Ministry of Education, Science, Sports and Culture of Japan. We thank Dr Hideo Yagita for donating the antibodies against mouse β integrin. We are grateful to Dr Nicholas J. Severs (The National Heart and Lung Institute, U.K.) for his advice on English usage.

References

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Circ Res. 1999;85:1046-1055
doi: 10.1161/01.RES.85.11.1046
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Materials and Methods

Surgical and tissue preparation

Myocardial infarction was induced in ninety-eight 9-week-old male Wistar rats by ligation of a branch of the left coronary artery as previously described. In the sham-operated group, the ligature was not tightened. This study was conducted in accordance to the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985), and the "Rules and Regulations of Animal Research, Kyoto Prefectural University of Medicine".

Hearts excised after coronary ligation at various times (6, 12, 24, 48 hours, day 3, 4, 8, 15, 30 and 60 post-ligation) were fixed by perfusion with 2% paraformaldehyde (PFA) in 0.01 mol/L phosphate-buffered saline (PBS, pH 7.4) via coronary arteries (5 minutes). Hearts were then transversely sectioned and immersion-fixed in 2% PFA at 4°C (2 hours). After gradual infiltration with Tissue-Tek OCT compound (Miles), the specimens were frozen in liquid nitrogen. For immunohistochemistry of β₁-integrin, excised hearts (48 hours, day 4, 15, and 90 post-ligation) were snap frozen without fixation.

Antibodies

The following antibodies were used: rabbit polyclonal antibody (pAb) to Cx43 (1:500, Zymed Laboratories); mouse monoclonal antibody (mAb) to Cx43 (1:500, Chemicon); mouse mAb to desmoplakin I+II (1:10, Boehringer Mannheim); mouse mAb to pan-cadherin (1:500, Chemicon); rabbit pAb to laminin (1:50, ICN Biomedicals); and hamster mAb to β₁-integrin (1:300; a kind gift from Dr. H. Yagita, Juntendo University School of Medicine). For detection of primary antibodies, we used Texas Red (TR)-labeled antimouse or anti-rabbit IgG (Vector Labs), fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG (Dako) or anti-mouse IgG (Cappel/Organon Teknika), biotinylated anti-hamster IgG (Vector) and FITC-labeled streptavidin (Vector). The antibodies against mouse IgG were adsorbed against normal rat serum. TR-X-labeled phalloidin (1:200, Molecular Probes) was used for detection of F-actin.
**Immunofocal microscopy**

Serially sliced 20-μm-thick frozen sections were fixed with ethanol (PFA-fixed hearts) or acetone (unfixed hearts) at -20°C (10 minutes), dried and rinsed in 5% skim milk in PBS (15 minutes), before washing in 0.1% Triton X-100 in PBS (15 minutes). Double-immunolabeling was performed using antibodies and reagents in the following sequence: (i) overnight incubation with a mixture of two of the above primary antibodies at 4°C; and (ii) incubation with a mixture of appropriate FITC- and TR-labeled secondary antibodies (1:200-300) at 37°C (2 hours). In some cases, biotinylated secondary antibodies and FITC-labeled streptavidin (1:100) were used. Specimens were mounted in Vectashield (Vector). Negative controls in which one of the primary antibodies was excluded were performed to rule out cross reactivity.

Immunolabeled sections were observed using a confocal laser scanning microscope (FluoView, Olympus). An argon/krypton laser system and an oil-immersion objective lens (PlanApo x60, N.A.=1.4, Olympus) were used. Simultaneous images (800 pixel x 600 pixel with 12 bits, each) of FITC and TR labels were recorded.

All figures were transverse optical sections of the left myocardium in which the cardiomyocytes were longitudinally sectioned. In the following, we use the terms "borderline" and "vicinity" to discriminate cells located differently with reference to the infarct as previously described. Briefly, a "borderline" cardiomyocyte has a longitudinal cell border facing the infarcts, leaving a stump, and adjoins a neighboring cardiomyocyte with an intercalated disk at the opposite longitudinal border. "Vicinity" cardiomyocytes locates the myocardial tissues surrounding the "borderline" cardiomyocytes at sites opposite the myocardial infarct. "Borderline" cardiomyocytes were recognized by TR-Phalloidin staining of the same or serially sliced sections in immunofluorescence, and/or by hematoxylineosin staining.

**Morphometry**

Each confocal image was collected using one-time zooming (field: 265 x 200 μm²).

We classified longitudinal cell boundaries into three categories according to their sites with
reference to the infarct, i.e. those between cells or sites in each of the three following categories: infarcted cardiomyocytes (I), stumps (S), and "vicinity" cardiomyocytes (V) which existed at less than 265 μm apart from the stump. Data were collected from "I" at 6 and 48 hours post-ligation, from "S" and "V" at six time points (6 and 48 hours, days 4, 8, 15 and 60 post-ligation), and from longitudinal cell boundaries between sham-operated left ventricular cardiomyocytes (Sham). The "black level" was constantly maintained to ensure that the outlines of individual cardiomyocytes were slightly visible. We adjusted the "gain" control using right ventricles as the control because they were less likely to be influenced by occlusion of the left coronary artery compared with the left ventricles. As a result, all pixel intensities for Cx43, desmoplakin and cadherin at each cell boundary of infarcted and sham-operated hearts were below the 4096-level scale. The digital images were converted into 8-bit images (256 level) before analysis by NIH Image (Wayne Rasband, NIH). A binary overlay was created automatically by a designated threshold of 50 on the 255-point gray scale to eliminate the background cell outlines (less than 30-point). Each longitudinal cell boundary was enclosed manually and the binary overlaid area-of-interest was measured.

For assessment of changes in the expression of Cx43, desmoplakin and cadherin at the cell boundary after myocardial infarction, we compared the immunopositive area of Cx43, desmoplakin and cadherin per unit area of intercalated disk ("I" and "V") or stump ("S") at various time points. The similar parameter has previously been used in Cx43 analysis of intercalated disks in human hearts. However, we could not apply en face viewed intercalated disks from transversely sectioned tissues. We therefore measured the immunopositive area in the enclosed field and cell width at each cell-cell and cell-matrix boundary. A certain area of the en face viewed intercalated disk [cell width x optical section thickness (0.5 μm on theoretical grounds)] can be obtained from a single optical section. Because the optical section thickness is fixed and intercalated disks were randomly cross-sectioned in our samples, the mean of immunopositive area/cell width reflects the amount of junctional proteins per unit area of en face viewed intercalated disks. Data were collected from 3-8 hearts for each time interval and 20-47 cell termini were actually analyzed for each group (total 1119 readings). Concerning "S", all cell processes at the stump were included,
and the cell width was measured at the base of cell processes. Finally, all data were converted so that the mean of each datum on Cx43, desmoplakin and cadherin of the "Sham" category was 100%. Using StatView software (Abacus Concepts Inc.), data were analyzed by two-factor factorial ANOVA and according to the respective time points. Significant differences between any two groups in all combinations (total 447 combinations) were defined by $P < 0.05$ using the Scheffé test (Table).

The expression of $\beta_1$-integrin at stumps was analyzed at 4 time points (48 hours, days 4, 15 and 90 post-ligation) and compared with that at the myotendinous junction of sham-operated left ventricular papillary muscles.

**Electron microscopy**

Excised hearts (48 hours, day 3, 4, 8, 15 and 60 post-ligation; 6 hearts for each time) with/without arrest by perfusion of cardioplegic solution were fixed by perfusing 3% glutaraldehyde and 2% PFA in 0.1 mol/L phosphate-buffer (pH 7.6) via coronary arteries. After cutting into small blocks, specimens were immersion-fixed in the fixative at 4°C (2 hours). The specimens were postfixed with OsO$_4$, treated with uranyl acetate and dehydrated before embedding in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination under an electron microscope (JEM 1010, JEOL). To obtain longitudinal edges of cardiomyocytes facing the infarct, we observed serial sections at 0.5-μm intervals.
Test results between CX43 and desmosplakin, CX43 and cadherin, or desmosplakin and cadherin in each category are shown. Statistical analysis was carried out for each time point, and the significant difference is denoted by P < 0.05 using the Scheffe test.

Comparisons were made with sham-operated carotidomyocardies (Sham). Following 24h and the number of measured areas (pixels x pixels) per cell width and of "Ventricular" cardiomyocytes (V). Values are the means of percentages of immunopositive areas (pixels) at slumps (S).

CX43, DP and Cad indicate connexin43, desmosplakin and cadherin, respectively.

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Table 1. Morphometry

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**Note:** The table is presented with the given data points. Further analysis or calculation requires additional context or specific data points that are not fully visible in the image.