Spatiotemporal Development and Distribution of Intercellular Junctions in Adult Rat Cardiomyocytes in Culture

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Abstract—The mode of development of the intercalated disk (ID) is largely unknown, and the hypothesis was tested that the assembly of cell adhesion junctions may precede the formation of gap junctions (GJ) in developing ID in adult rat cardiomyocyte (ARC) in long-term culture. Immunostaining for connexin 43 (Cx43) and for cell adhesion junction proteins (N-cadherin, catenins, and desmoplakin) in single- and double-label techniques was analyzed and quantified by confocal and electron microscopy. All proteins investigated disappeared 48 hours after ARC isolation and reappeared parallel to redifferentiation of ARC. The newly formed ID, observed after 5 days, showed the presence of N-cadherin, catenins, and desmoplakin, low levels of Cx43, and absence of ultrastructurally discernible gap junctions. A progressive incorporation of Cx43 within ID was observed after 6 days, when cell adhesion junction proteins were already organized as zipper-like structures. Quantitative confocal analysis revealed a progressive augmentation of the fluorescence intensity of Cx43, associated with an increase in both the number and size of GJ, resulting in a substantial increase in the percentage of total GJ length per reassembled ID from 1.67% (day 6) to 15.58% (day 12). In the present study, we show that (1) the formation of the ID can be followed in ARC in culture and (2) the assembly of the adhering type of junction is the prerequisite for subsequent GJ formation within the ID. These findings may have clinical relevance in elaborating strategies for using myocardial grafts and for the potential restoration of GJ communication in cardiac diseases. (Circ Res. 1999;85:154-167.)

Key Words: gap junction ▪ fascia adherens ▪ desmosome ▪ intercalated disk ▪ development

Cardiac muscle cells are interconnected by 3 distinct types of intercellular junctions: gap junctions (GJ), fasciae adherentes, and desmosomes—located in a specialized portion of the plasma membrane, the intercalated disk (ID). GJ form the low-resistance pathway that enables rapid conduction of cardiac action potential throughout the myofibers, thereby synchronizing contractions of the heart. The fascia adherens and desmosome belong to the group of adhering junctions and are responsible respectively for attachment of the contractile filaments and the intermediate filaments to sites of intercellular attachment.

The molecular makeup of GJ, fascia adherens, and desmosome is now well characterized. However, the mechanism by which different junctional molecules during the assembly of the ID are sorted in a precise spatial and sequential manner to sites of function is still poorly understood. One basic approach to study the formation of ID is the cardiomyocyte in long-term culture, a model that has been used with increasing sophistication in recent years for different issues of heart cell research. However, little is known about the ability of these cells to form new intercellular contacts and to reassemble the ID. Therefore, we explore in detail, the time course of appearance and distribution of ID-associated proteins and the ultrastructural sequential patterns of intercellular junction formation in ARC in culture. In the present study, we tested the hypothesis and show that the formation of the adhering type of junction is essential for the stable cell-cell contact and is the prerequisite for subsequent GJ formation within the ID.

Materials and Methods

Isolation and Culture of Cells

Experiments were performed according to a protocol approved by the Regierungspräsidium, Darmstadt, Germany. Wistar rats (7 to 8 weeks old; Süddeutsche Versuchstierzucht, Tuttingen, Germany) were deeply anesthetized with ether. The heart was excised and perfused retrogradely with a Ca²⁺-free perfusion buffer (PB) containing (in mmol/L) NaCl 110, KCl 2.6, MgSO₄ 1.2, KH₂PO₄ 1.2, glucose 11, and HEPES 10 (at 37°C, pH 7.4, gassed with 95% O₂/5% CO₂). Perfusion was then switched for 20 minutes to 0.03% collagenase (CLS 2, Worthington Biochemical Corp), 0.004% pronase (Boehringer), 0.005% trypsin (Sigma), and 0.04 mmol/L CaCl₂ in PB. The ventricles were minced in the collagenase solution containing 1.2% BSA at 37°C for 10 minutes, filtered through a nylon mesh, and centrifuged at 8g for 3 minutes. The pellet was
washed in PB containing 0.1 mmol/L CaCl₂ followed by separation in 33% Percoll (Pharmacia). Calcium was then added stepwise to a concentration of 1.0 mmol/L. The cells were resuspended in medium 199 (Sigma) containing 5 mmol/L creatine, 2 mmol/L L-carnitine, 5 mmol/L taurine, 0.1 mmol/L insulin, 10 mmol/L cytosine arabinoside, 100 IU/mL penicillin-streptomycin, and 10% FCS, plated on culture chamber slides (Nunc), coated with 5 mg/mL laminin (Sigma) at 2×10⁴ cells/well, and incubated in a 95% O₂/5% CO₂-incubator at 37°C. After 3 hours, the medium was replaced with the same fresh medium, and the cells were cultured up to 15 days as previously described.¹ The cells were harvested and investigated daily during the first week and every 3 days during the second week in culture. The results reported are based on 7 highly reproducible long-term cultures: 2 cultures for single-labeling experiments, 3 cultures for double-labeling procedures and ultrastructural analysis, and 2 cultures for quantitative immunofluorescence.

**Immunocytochemistry**

ARC were fixed for 10 minutes with 4% paraformaldehyde and permeated for 15 minutes with PBS containing 0.05% Triton X-100. Cells were exposed for 10 minutes in 0.1% BSA, followed by incubation with the corresponding antibodies in single- or double-staining procedures.

**Antibodies**

Monoclonal (clone GC-4) and polyclonal antibodies against N-cadherin, monoclonal anti-plakoglobin (clone 15F11), polyclonal anti-α-catenin, or polyclonal anti-β-catenin, were purchased from Sigma. Desmosomes were stained with monoclonal (clone DP 1&2-2.15, Boehringer) or polyclonal (SAD 3120, NatuTec) antibodies against desmoplakin. Connexin (Cx)43 was detected with a monoclonal antibody (clone 1E9) raised against amino acids 252 to 292.

![Figure 1](http://circres.ahajournals.org/) Freshly isolated ARC. 3-D distribution of the immunolabeled fasciae adherentes for N-cadherin (A and B), desmosomes for desmoplakin (C), and GJ for Cx43 (D). Myofibrils are stained red with phalloidin.
270 of rat Cx43 (Biotrend). Monoclonal antibody against myomesin (clone B-4) was a generous gift from Dr H.M. Eppenberger (Institute of Cell Biology, ETH, Zürich, Switzerland).

**Single Staining**

The cells were incubated with the monoclonal antibody for 12 hours at 4°C. After repeated washes with PBS, the cells were incubated for 2 hours at room temperature with biotinylated donkey anti-mouse IgG (Dianova) followed by Cy2-conjugated streptavidin (Rockland). Specificity of the labeling was confirmed by omission of the primary antibody. The nuclei were stained with 7-aminoactinomycin D (Molecular Probes). F-actin was fluorescently stained using TRITC-conjugated phalloidin (Sigma).

**Double Staining**

The cells were incubated with primary monoclonal antibodies and then incubated with biotinylated donkey anti-mouse IgG, followed by Cy2-conjugated or Cy3-conjugated streptavidin. The cells were washed and incubated with polyclonal antibodies, followed by FITC (Dianova) or Cy3-conjugated goat anti-rabbit IgG (Chemicon International). The following controls in the double-labeling procedure were used: (1) omission of both primary antibodies, (2) alternating of the detection system in single-labeling experiments (e.g., using mouse monoclonal followed by anti-rabbit secondary antibodies), and (3) reversing the order of primary antibodies.

**Confocal Microscopy**

The cells were examined by a laser scanning confocal microscope (Leica TCS 4D) equipped with an argon/krypton mixed gas laser, which allows an improved signal separation of FITC or Cy2 from TRITC or Cy3 fluorescence. Series of confocal optical sections (from 10 to 50) were taken through the depth of ARC at 0.5- to 1-μm intervals by using either a Leica Neofluar ×40/1.0 or Leica Planapo...

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**Figure 2.** ID region in freshly isolated ARC and after 24 to 48 hours in culture. A, 0 hours, An abundance and a plicate configuration of the fasciae adherentes (arrowheads). Arrow indicates a surface-located GJ. B, 24 hours, Accumulation of dense material (arrows) of internalized junctional plaques at the end of myofibrils. C, 48 hours, Disappearance of the electron-dense material from the former region of the ID. Arrow indicates an annular GJ profile. Bar = 1 μm in panels A through C, D, Cx43 immunofluorescence is still abundantly present (arrows) at 24 hours. E, Almost complete disappearance of Cx43 after 48 hours. Nuclei are red. Bar = 10 μm.
×63/1.4 objective lens. Each recorded image was taken using dual-channel scanning and consisted of 512×512 pixels.

To improve image quality and to obtain a high signal to noise ratio, each image from the series was signal-averaged. After data acquisition, the images were transferred to a Silicon Graphics Indy workstation (Silicon Graphics) for restoration and 3-dimensional (3-D) reconstruction using Imaris, the 3-D multichannel image processing software (Bitplane, Zürich, Switzerland). The principles of this method have been previously described.2 In this technique, the optical sections of ARC, simultaneously labeled with different fluorochromes, could be viewed individually or superimposed to reconstruct the entire labeled structures in a complete 3-D distribution.

Quantitative Analysis of Fluorescence Intensity

Two cultures were used for quantification of the fluorescence intensity (FI) of N-cadherin and Cx43. After fixation and permeation (see Immunocytochemistry), the cells were exposed to 0.5% BSA for 15 minutes and then incubated sequentially with (1) polyclonal anti–pan-cadherin (1:500), (2) anti-rabbit IgG-FITC (1:100), (3) monoclonal anti-Cx43 (1:500), and (4) anti-mouse IgG-TRITC (1:100) for 12 hours at 4°C each step. Repeated washes with PBS were done after each step of the immunolabeling procedure. The order of the primary antibodies had no effect on the result. ARC exposed to PBS instead of primary antibodies, but incubated sequentially with both detection systems, served as a negative control and was run in parallel during each quantitative experiment. All process-

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**Figure 3.** 3-D confocal images of double-labeled ARC for N-cadherin (A, D, and G) and Cx43 (B, E, and H). Representative histograms show the distribution of FI of both proteins per voxels from the respective boxed areas in panels A, B, D, E, G, and H at 0 hours (C), 24 hours (F), and 48 hours (I) after isolation.
ing and immunolabeling procedures were done under identical conditions for all groups.

Quantification of N-cadherin and Cx43 was performed by measurements of FI using simultaneous dual-channel confocal scanning. The confocal settings had been standardized for all experimental groups to ensure that the images collected demonstrated a full range of FI from 0 to 255 intensity levels and were kept constant for recording of data in all measurements. Optical sectioning was done through the depth of ARC from the “ventral” to “dorsal” membrane using a ×40 objective (Leica, Neofluar, numeric aperture 1.0). The number of collected images was calculated as axial thickness (in μm) multiplied by factor 5, which in a field size of 100 × 100 μm and in a 512 × 512-pixel format yielded a voxel size of 0.2 × 0.2 × 0.2 μm or a voxel volume of 0.008 μm³. Twelve randomly selected fields (size 100 × 100 μm) comprising 1 to 3 ARC were investigated per each time point (ie, 2 cultures × 6 fields per culture). Collected series of confocal images were additionally magnified 5 times and inspected in microscopy, several maximum signal-averaged 3-D regions of ARC were magnified 5 times and inspected in x-y-z dimensions to ensure that all voxels were in the region of interest (ie, dissociated ID, redeveloped ID, perinuclear region, or pseudopods) and then saved as separate images to directly display the histograms of FI distribution in the Voxel Shop Program (Bitplane, Zürich, Switzerland) or converted into Macintosh Excel data for statistical analysis. Representative histograms showing the results of single measurements of FI in well-defined 3-D regions of interest are depicted in Figures 3 and 10. Each measured region encompassed a volume of 125 ± 15 μm³ and included 15 654 ± 1875 voxels (n = 676 measurements). The value of FI in individual measurements was expressed as mean FI (in arbitrary units) per voxel. The average value of FI per optical field was calculated from 3 to 8 measurements. The average integrated FI value per time point was calculated from 80 to 96 measurements from 12 randomly selected fields and was further used for comparison of quantity of N-cadherin or Cx43 between groups. It should be emphasized that the FI measurements do not provide absolute values of the total cellular content of the investigated proteins or the dynamics of their synthesis or degradation. However, these measurements may be regarded as useful estimates of the relative quantities in well-defined regions of a single cell and allow comparisons and conclusions as to whether their quantity is changed at the different time intervals. The reproducibility of the quantification was assessed by analyses of selected fields performed by two investigators, who obtained remarkably similar values of FI.

One-way ANOVA on ranks was used to test the significant changes in FI, followed by analysis with the Bonferroni t test. Results are reported as mean ± SD. Differences between groups were considered significant at P < 0.05.

### Table 1. Quantitative Immunofluorescence (Arbitrary Units) of N-Cadherin and Cx43 in ARC Cultured for 0 to 48 hours

<table>
<thead>
<tr>
<th></th>
<th>0 Hours (n=12)</th>
<th>24 Hours (n=12)</th>
<th>48 Hours (n=12)</th>
<th>Negative Control (n=48)</th>
</tr>
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<tbody>
<tr>
<td>N-cadherin</td>
<td>76.5 ± 16.9</td>
<td>74.5 ± 20.1</td>
<td>15.5 ± 5.5</td>
<td>1.43 ± 0.39</td>
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<tr>
<td>Cx43</td>
<td>28.4 ± 8.5</td>
<td>25.2 ± 8.1</td>
<td>4.5 ± 2.5</td>
<td>1.22 ± 0.66</td>
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*P < 0.05 compared with 0 hours.

**Figure 4.** ARC, 4 days, labeled for desmoplakin (A) and for N-cadherin (B). Both proteins are accumulated in the perinuclear area and in the pseudopods (arrow in panel A) in a striped pattern. Nuclei are red. Bar = 10 μm.

**Electron Microscopy**

ARC were fixed for 2 to 4 hours in 0.1 mmol/L sodium cacodylate plus 7.5% sucrose with 3% glutaraldehyde and postfixed in 1% osmium tetroxide for 1 hour. After rinsing in a series of ethanol, the samples were embedded in Epon following routine methods. Thin sections were poststained with uranyl acetate and Reynolds lead citrate and photographed with a Philips CM10 electron microscope. GJ profile length was measured in randomly photographed ARC to determine the relative number and size of GJ per unit ID length. At least 10 randomly selected ID cut en face and 10 cut perpendicular to the substratum were selected for morphometric analysis from each group. Initially, ID were photographed at low magnification to measure their total length, then all portions of the ID containing GJ profiles were photographed again for further analysis at a final print magnification of ×30 000.
Figure 5. ARC, 5 days. A, Ultrastructure of the newly formed ID (arrows). B, Scarce plaque-like structures at the ID area (arrow); arrowhead indicates sarcoplasmic reticulum. C, Adherens-like sarcoplasmic condensations (arrows) of the sarcolemmas of 2 ARC. Bar=1 μm for panel A; 0.5 μm for panels B and C. N-cadherin (D) and desmoplakin (E) are localized at the ID in a continuous linear pattern. Nuclei are red. Double immunolabeling for plakoglobin (red) and α-catenin (green) (F) or for pan-cadherin (red) and Cx43 (arrowhead, green) (G). Notice the colocalization of α-catenin with plakoglobin (yellow) at the ID (arrows) in panel F. Bar=10 μm.
Figure 6. ARC, 6 to 7 days. Single confocal section (A) and 3-D distribution (B) of Cx43 (green, arrows) and of pan-cadherin (red) in 6-day ARC. 3-D image of double immunolabeling for Cx43 (red) and for α-catenin (green) (C) or β-catenin (green) (D) in 7-day ARC. Bar=10 μm. E, ARC, 6 days, sectioned in parallel to the culture substratum, shows a single GJ profile (arrow), neighbored at both sides by large segments of adherens-like “zippers” (arrowheads). F, ARC, 7 days, sectioned transversely to the substratum, shows GJ (arrows) surrounded by multiple adherens plaques (arrowheads). Bar=0.5 μm.
Results

Disassembly of Intercellular Junctions
Immediately after isolation, the majority of the cardiomyocytes were rod-shaped, retaining the step-like appearance of the ID and the abundance of the intercellular junctions characteristic of intact tissue. Figures 1A through 1D illustrate the 3-D distribution of the adherens junctional (N-cadherin), desmosomal (desmoplakin), and GJ (Cx43) proteins at the freshly dissociated ID. As established in the present study and previously,4 dissociation of myocytes involves separation of the membranes comprising the ID such that the adherens junctions are cleaved symmetrically, whereas the separation of GJ occurs as a result of tearing out of these structures such that the GJ remain in toto as annular profiles in the cytoplasm or as intact bimembranous surface-located pentalaminar structures (Figure 2A). Despite this vulnerable step in separation of the ID, most of the cells retained the ultrastructural features of intact cardiomyocytes.

During subsequent maintenance in culture, ARC underwent a smoothing-over of the ID region, involving the internalization of the fasciae adherentes (Figure 2B) and the replacement of the step-like appearance of the disk with a smoothly contoured plasma membrane (Figure 2C). At 24 hours in culture, immunolabeling for Cx43 revealed numerous GJ, located at cell margins (Figure 2D). A decline in Cx43 immunofluorescence was observed in ARC maintained in culture for more than 24 hours, culminating in an almost

Figure 7. ARC at day 9. Single labeling for plakoglobin (A) and N-cadherin (B) and double labeling for Cx43 (red) with pan-cadherin (C) or desmoplakin (D). Notice the appearance of plakoglobin and N-cadherin in a continuous pattern, whereas desmoplakin appears as fluorescent dots (in panel D). In panels A and B, nuclei are red. Bar=10 μm.
complete disappearance of the fluorescent signal at 48 hours (Figure 2E).

To more precisely determine whether, and, if so, to what extent, the junctions forming the ID are degraded after myocyte dissociation, a quantitative analysis was performed. For this purpose, ARC were double-labeled for N-cadherin and Cx43, and the FI of these proteins was measured by using dual-channel quantitative immunofluorescence of the ID regions in a 3-D imaging mode. Figure 3 shows the overall distribution of the immunolabeled fasciae adherentes and GJ, complemented with representative recordings of the FI distribution in well-defined regions of the ID in ARC from 0 to 48 hours in culture. Table 1 shows the average integrated values of FI of N-cadherin and Cx43 per each group. At 24 hours, a slight decrease in FI of N-cadherin and Cx43 was observed. However, a pronounced and statistically significant ($P<0.05$) diminution in FI of both proteins occurred at 48 hours. These results indicate that Cx43 and N-cadherin appear to persist 24 hours after cell isolation, whereas the following period in culture demonstrates the capacity of ARC to degrade the internalized GJ and cell-cell adhesion junctions.

Figure 8. 3-D and subcellular organization of myofibrils and ID in ARC at day 12. A, Labeling for F-actin and myomesin shows a clear cross-striation in the perinuclear regions. B, Double labeling for pan-cadherin (green) and Cx43 (red) reveals the distribution of GJ in a dispersed pattern across the ID. The arrow points to the absence of Cx43 at the ID. C, Different populations of GJ (small and large arrows). D, GJ (arrows) alternates with desmosomes (arrowheads). E, Myofibrils are oriented and terminate in a fascia adherens-like dense plaque (white arrow). GJ are indicated with black arrows. F and G, Large ribbon-like GJ profiles (>5 μm) (arrows). Bar=30 μm for panel A; 10 μm for panels B and C; 0.3 μm for panel D, and 0.5 μm for panels E through G.
Figure 9. ARC after 2 weeks in culture. A, 3-D images of N-cadherin (green) at 15 days showing a high level of confluency and numerous ID structures. B, High-magnification confocal image shows the stair-like appearance of the fascia adherens, revealed by N-cadherin. Notice a clear cross-striation of actin fibers (red). C, Corresponding with panel B findings by electron microscopy, actin filaments insert into the dense filamentous plaque of the fascia adherens. D, Well-developed interdigitating ID. E, ID in adult rat myocardium in situ. Bar=10 μm for panels A and B; 1 μm for panels C, D, and E.
Redevelopment of ID-Like Structures
The ensuing period in culture (3 to 4 days) includes cell growth, extensive spreading on the substratum, and the formation of pseudopods. At this stage, desmoplakin (Figure 4A) and N-cadherin (Figure 4B) were observed to accumulate in a striped pattern in the perinuclear region and in the pseudopods, where α-catenin and plakoglobin could be also identified, but only a weak fluorescent signal was detected for Cx43.

Figure 10. 3-D confocal images of double-labeled ARC for N-cadherin (A, D, and G) and Cx43 (B, E, and H) at 4 days (A and B), 6 days (D and E), and 12 days (G and H). Representative histograms show the distribution of FI per voxels from the respective boxed regions of panels A, B, D, E, G, and H.

TABLE 2. Quantitative Immunofluorescence (Arbitrary Units) of N-Cadherin and Cx43 in ARC Cultured for 4 to 12 days

<table>
<thead>
<tr>
<th></th>
<th>4 Days (n=12)</th>
<th>6 Days (n=12)</th>
<th>9 Days (n=12)</th>
<th>12 Days (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-cadherin</td>
<td>60.9±10.2</td>
<td>74.8±15.4</td>
<td>85.6±13.8</td>
<td>81.6±10.4</td>
</tr>
<tr>
<td>Cx43</td>
<td>6.3±3.9*</td>
<td>8.8±4.1*</td>
<td>21.7±5.6</td>
<td>31.2±4.0</td>
</tr>
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</table>

*P<0.05 compared with 12 days.
After 5 days, a progressive increase in cell size and the extension of the pseudopods were accompanied with the formation of new ID (Figure 5A), ultrastructurally characterized by closely apposed plasma membranes, the presence of fibrillar connections in the intercellular gap, and the appearance of scarce subplasmalemmal plaque-like structures (Figure 5B). After establishment of this type of connection between ARC, the intercellular contacts extended to larger areas, and the electron-dense submembranous plaques became conspicuously prominent and appeared as symmetrically clustered sarcoplasmic condensations (zipper-like structures) along the opposite plasma membranes of neighboring cardiomyocytes (Figure 5C). The early ID followed a rather straight course between cells, and there was little structural evidence of actin filament insertion or anchoring into these cell adhesion junctions.

By immunofluorescence, desmoplakin (Figure 5D), N-cadherin (Figure 5E), α-catenin, and plakoglobin (Figure 5F) distinctly colocalized at the newly formed ID. By contrast, Cx43 was found only at very low levels (Figure 5G). In addition, there was an almost complete absence of ultrastructurally discernible GJ in these early ID. These data indicate an appearance of cell adhesion proteins earlier than Cx43.

After 6 to 7 days, when adhesion junction-specific proteins, including N-cadherin (Figure 6A and 6B), α-catenin (Figure 6C), and β-catenin (Figure 6D), were already organized within the ID, Cx43 progressively accumulated within these structures (Figure 6A through 6D), finally leading to the formation of typical pentalaminar structures, representing intact GJ, as were seen under the electron microscope (Figure 6G and 6H).

After 9 days in culture, the ID between ARC, as revealed by plakoglobin (Figure 7A) and N-cadherin (Figure 7B and 7C), spread over extensive segments of their sarcolemma. In comparison with the linear staining pattern of adherens junction proteins, desmoplakin, representing individual desmosomes, now appeared as fine fluorescent dots (Figure 7D). At this stage, immunolabeled GJ for Cx43 were observed more frequently, compared with 6 to 7 days, and they were uniformly distributed in a punctate pattern along the ID (Figure 7C and 7D).

**ID in Redifferentiating ARC**

At 12 days in culture, most ARC showed perinuclear foci of newly forming myofibrils exhibiting a distinct cross-striated sarcomeric pattern after myomesin labeling (Figure 8A), which is a characteristic marker for mature sarcomeres. At this time point, the ID showed further development, including numerous and polymorphic GJ (Figure 8B and 8C), a distinct segregation of cell adhesion junctions into desmosomes (Figure 8D) and fascia adherens (Figure 8E), and a clear insertion of the actin filaments into the fascia adherens (Figure 8E). Furthermore, at the electron-microscopic level, large ribbon-like GJ, which are typical of adult ventricular cardiomyocytes in situ, could also be observed (Figure 8F and 8G).

After 2 weeks, as organized sarcomeres increased, the ID became spatially more complex. The process of junctional and myofibrillar differentiation is illustrated in Figure 9. Cardiomyocytes maintained in culture for more than 2 weeks showed a high level of confluency (Figure 9A) and well-developed junctions with features of a classic ID (Figure 9B). Dense plaques of the fascia adherens were well-developed, and actin filaments terminated directly into these plaques (Figure 9C). The appearance of highly organized ID (Figure 9D), closely resembling those in situ (Figure 9E), was coupled with the development of rhythmic beating activity, which further enhanced the development of highly differentiated contractile and junctional structures, characteristic of the mature ARC phenotype in intact myocardial tissue.

**Quantitative Analysis of ID Formation**

We determined quantitatively the time course of Cx43 and N-cadherin incorporation into developing ID. Figure 10 shows the 3-D view of double-immunolabeled ARC for N-cadherin and Cx43, including the corresponding representative histograms of FI distribution. Results are provided in Table 2. At 4 days in culture, cell-cell contacts were rarely seen; however, inspections of different cellular compartments, such as cell body or pseudopods (Figure 10A through 10C), revealed high levels of N-cadherin FI and low values of Cx43 FI. At day 6, the redeveloped ID showed high levels of N-cadherin FI and low signal for Cx43 (Figure 10D through 10F). However, with increasing time in culture, these structures showed a progressive increase in the FI of Cx43 in that the mean value of FI increased by 247% from day 6 to day 9 and by 355% from day 6 to day 12 (P<0.05). By contrast, the changes in mean values of N-cadherin FI were not statistically significant. The values of FI of either N-cadherin or Cx43 at 15 days in culture did not differ from those at 12 days (not shown).

We next examined by quantitative electron microscopy whether increased FI of Cx43 at the ID, as a function of time, parallels with changes in the number and size of GJ. Table 3 shows that a progressive increase in both the number and size of GJ resulted in a substantial increase in the percentage of total GJ length per reassembled ID from 1.67% (day 6) to 15.58% (day 12) (P<0.05), thus confirming the immunofocal observations.

<table>
<thead>
<tr>
<th>Time</th>
<th>Total ID Length, μm</th>
<th>Number of GJ</th>
<th>Total GJ Length, μm</th>
<th>Average GJ Length, μm</th>
<th>Percentage of GJ Length per ID Length</th>
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<td>6 days</td>
<td>145.6</td>
<td>8</td>
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<td>9 days</td>
<td>169.3</td>
<td>24</td>
<td>12.2</td>
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<td>12 days</td>
<td>179.1</td>
<td>30</td>
<td>27.9</td>
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<td>15.58</td>
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</table>

Kostin et al Development of Cardiac Intercalated Disks
Discussion

Descriptive electron-microscopic studies on cultured ARC showed that specialized junctions forming the ID in vivo can be regained in vitro. The results of the present study considerably extend these findings and provide a more detailed picture of the appearance of intercellular junctions and their proteins in ARC at the time of cell separation and during subsequent maintenance in culture.

Although the major goal in our study was to investigate the time course of appearance and distribution of the proteins involved in the reassembly of ID, some aspects of the fate of ID after cell dissociation should be noted. On the basis of electron-microscopic observations of dissociated ARC, Mazet et al proposed a GJ degradation concept referring to the progressive GJ endocytosis and inward migration of GJ vesicles, followed by their lysosomal degradation over a period of several hours after cell isolation. However, a more detailed study by Severs et al in rabbit and cat cardiomyocytes maintained for 15 to 22 hours in culture medium provided no structural evidence for movement of internalized GJ or of their degradation. The latter findings were confirmed in the present study, using confocal microscopy, which provides information on the 3-D overall distribution of GJ in an individual cardiomyocyte. Similarly, in ARC maintained for 24 hours in culture, we found the majority of GJ to be confined to cell termini. In addition, by using quantitative immunofluorescence, we found only a modest decrease in the FI of Cx43 at this time point. Nevertheless, an ultimate disappearance of immunolabeled GJ was observed, as expected earlier, in myocytes maintained in culture for more than 24 hours.

The “redifferentiation” model of ARC in culture has been used in many studies on myofibrillogenesis, cell-substrate interactions, and rearrangement of the cytoskeleton. After attachment and slow morphological transition from the elongated in vivo structures to a flat polygonal shape during dedifferentiation, ARC disassemble and/or degrade the contractile/cytoskeletal apparatus and, as shown in the present study, the ID structures. This is followed by subsequent regeneration of the myofibrillar and cytoskeletal apparatus and the restoration of mechanical and electrical coupling between redifferentiating cardiomyocytes.

We demonstrate in the present study that the formation of the ID can readily be observed in primary heart cell cultures. ARC do not divide or move on the substratum in culture; therefore, the formation of ID is achieved by formation of pseudopods and increasing the cell volume by a factor of ≈2, compared with the original volume. To elucidate the mode of development of ID, we have taken the advantage of the plasticity and strong tendency of ARC to communicate and to reassemble ID structures during the process of dedifferentiation-redifferentiation of cardiac phenotype in culture. A number of proteins were involved in the recovery of the dissociated ID: N-cadherin, catenins, and desmoplakin. All of these proteins colocalized in the cytoplasm in a characteristic striped pattern before clustering at the ID, suggesting an early formation of the protein complexes in the Golgi apparatus. Recent evidence supports the concept that the essential role of classic cadherins (of which N-cadherin, the principal protein of the cardiac fascia adherens, is an example) in the formation of homophilic cell-cell contacts interferes with the formation of functional cadherin/catenin complexes. The expression of α-catenin seems to be one of the prerequisites for cell adhesions, whereas β-catenin seems to be involved in early events of cell-cell adhesion, because it mediates the α-catenin/cadherin interaction. In agreement with these data, we found α-catenin and β-catenin consistently at the redeveloped ID.

The localization at the reassembled ID of adherens junctional (N-cadherin and catenins) and desmosomal components (desmoplakin) in a continuous linear pattern suggests that a temporal intermingling of these junctions occurs in spreading ARC. Nevertheless, at later stages of culture, we found a clear segregation of the fascia adherens and desmosome into separate junctions. Because plakoglobin is the major protein component common to both types of junctions, and because it is present at the ID, it is tempting to speculate that in cultured ARC plakoglobin may play a role in sorting desmosomal and adherens junction components. Recent evidence in support of this hypothesis has emerged from plakoglobin null mutant mice showing a severely affected architecture of the ID and a disturbed junctional differentiation.

On the basis of our observations, a temporal sequence for the development of ID in vitro is proposed in that the formation of adherens junctions is the prerequisite for subsequent progressive GJ formation within the ID. This implies that during the establishment of cell-cell contacts, transmembrane cadherins form a zipper-like structure, coupled to a cytoplasmic plaque of catenins, thus strengthening the cell-cell contact and providing enough close membrane apposition to allow the assembly of Cx43 into the GJ. This hypothesis would be in good agreement with the observations that antibodies for classic cadherins, transfection of cells with cDNA encoding cell adhesion molecules, and Ca2+ depletion, inhibiting cell-cell contact, significantly perturb the formation of GJ.

Moreover, ARC in long-term culture undergo distinct dedifferentiation steps and resemble in certain aspects embryonic or neonatal heart cells. Therefore, the mode of development of ID reported in the present study may give some clues about how the ID is formed during embryonic and postnatal heart development. It has been shown that in early mouse or rat myocardium, both the number and size of GJ are small but increase during development. By contrast, N-cadherin appeared in a pattern corresponding to an early ID, even before myofibrils could be observed. A recent quantitative study of developing rat or dog ventricles during perinatal growth of the heart has shown that adhesion junctions, providing additional clustering of GJ, quickly differentiate into definitive ID while GJ showed steady accumulation toward the nascent ID. These findings, suggesting that cell adhesion-rich zones act as foci for progressive GJ accumulation and preservation, are consistent with our hypothesis. In addition, the cell adhesion junction has also been shown to be an important determinant of the spatial patterning of the GJ during postnatal differentiation of human ventricular myocardium. Given that such close association
between intercellular junctions also exists in mature ID, this may have a potential clinical relevance in pathological situations such as the infarct border zone or regions of myofiber disarray in cardiomyopathies, which show localized disruptions to GJ distribution.24–26

The finding of the present study that GJ formation is promoted by cell-adhesion membrane apposition may also be important in (1) the feasibility of using grafts of myocyte suspensions to repair damage in the diseased heart27 and (2) synchronization of mechanical and electrical activity between native and donor regions of the atria in cardiac transplant recipients. Success in these instances depends on full and complete mechanical and electrical integration of the grafts of myocardium or myocytes with host myocardium by formation of ID and assembly of GJ.

References
Spatiotemporal Development and Distribution of Intercellular Junctions in Adult Rat Cardiomyocytes in Culture
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