Morphofunctional integration between skeletal myoblasts and adult cardiomyocytes in coculture is favored by direct cell-cell contacts and relaxin treatment

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Submitted 16 July 2004; accepted in final form 15 October 2004

The success of cellular cardiomyoplasty, a novel therapy for the repair of postischemic myocardium, depends on the anatomical integration of the engrafted cells with the resident cardiomyocytes. Our aim was to investigate the interaction between undifferentiated mouse skeletal myoblasts (C2C12 cells) and adult rat ventricular cardiomyocytes in an in vitro coculture model. Connexin43 (Cx43) expression, Lucifer yellow microinjection, Ca2+ transient propagation, and electrophysiological analysis demonstrated that myoblasts and cardiomyocytes were coupled by functional gap junctions. We also showed that cardiomyocytes upregulated gap junctional communication and expression of Cx43 in myoblasts. This effect required direct cell-to-cell contact between the two cell types and was potentiated by treatment with relaxin, a cardiotropic hormone with potential effects on cardiac development. Analysis of the gating properties of gap junctions by dual cell patch clamping showed that the copresence of cardiomyocytes in the cultures significantly increased the transjunctional current and conductance between myoblasts. Relaxin enhanced this effect in both the myoblast-cardiomyocyt and myoblast-cardiomyocyte cell pairs, likely acting not only on gap junction formation but also on the electrical properties of the preexisting channels. Our findings suggest that myoblasts and cardiomyocytes interact actively through gap junctions and that relaxin potentiates the intercellular coupling. A potential role for gap junctional communication in favoring the intercellular exchange of regulatory molecules, including Ca2+, in the modulation of myoblast differentiation is discussed.

gap junctions; connexin43

CELL TRANSPLANTATION FOR CARDIAC REPAIR (cellular cardiomyoplasty, CCM) is currently emerging as a potential novel approach for the treatment of postinfarct ventricular dysfunctions and progressive heart failure (21). The limited capability of myocardium to regenerate after injury and organ donor shortage have triggered during the past decade a constant search for the identification of the appropriate cell type capable of increasing the contractile elements and replenishing the damaged myocardium. A number of cell-grafting techniques, including the use of cardiomyocytes, skeletal myoblasts, and stem cells with cardiomyogenic and neoangiogenetic potential, have shown promise in several animal models (10). In particular, immature cardiomyocytes engrafted into postinfarcted myocardium may undergo a normal differentiation program and form intercalated discs with host myocardium, suggesting electro-mechanical coupling (24). Moreover, pluripotent mesenchymal, embryonic, and bone marrow stem cells, as well as endothelial progenitor cells, are able to express cardiomyocyte-like features and differentiate into functionally active cardiomyocytes, either upon implantation into the postischemic myocardium or upon coculture with cardiomyocytes (10, 24). However, several questions have been raised regarding limitations of the actual therapeutic value of cardiomyocytes and stem cells for the repair of the infarcted myocardium (4). Issues of concern are the allogenic origin of these cells, which requires immunosuppressive treatment; the high susceptibility to ischemia; the low yield of cardiomyocytes; and the potential for tumor development and/or non-muscle tissue formation of stem cells. Because of these problems, a great deal of interest has developed regarding the use of skeletal muscle myoblasts (6, 18), and several clinical trials are under way to examine the efficacy of using these cells for cardiac repair (9). These cells have many desirable advantages in the field of cardiomyoplasty, such as high tolerance to ischemia, high proliferative potential, and an autologous source. Interestingly, skeletal myoblasts express the gap junction protein connexin43 (Cx43) (1), which is considered a marker for cardiomyocyte intercellular coupling. This protein is present only in replicating myoblasts, however, and is downregulated soon after terminal differentiation to skeletal myotubes has been achieved. Indeed, the expression of Cx43 has been demonstrated to be critical for the coordinated cell behavior required for myoblast differentiation (23, 28). On the other hand, if skeletal myoblasts must be considered as repair cells for the infarcted heart, the implants must be integrated anatomically with the preexisting myocardium through the formation of gap junctions and must develop cardiomyocyte-like features to avoid alterations in the electrical conduction of the heart. The use of this tissue is still controversial. There are several studies showing that the implanted myoblasts undergo the normal skeletal maturation.

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program by forming myotubes, with no functional connections to the host myocardium (15, 25). However, evidence also exists that myoblasts and even myotubes may transdifferentiate into cardiomyocyte-like elements and develop apparent contacts with native cardiomyocytes via putative gap junctions (11, 19, 26, 30). It is possible that the lack of intercellular coupling between myoblasts and cardiomyocytes may be hindered by local environmental stresses that develop at the site of cell injection, including scar tissue formation, ischemic injury, and free radical generation. Thus prevention or limitation of these factors may greatly improve integration of the grafted cell with the host tissue. For instance, heat shock pretreatment of grafted myoblasts has recently been shown to improve their survival and myocardial integration after transplantation (29).

The present in vitro study was designed to provide further insights into the mechanisms controlling the intercellular communications between cardiomyocytes and skeletal myoblasts, with the aim of identifying the most suitable conditions for myoblast grafting and integration with the host myocardium. To achieve this purpose, mouse C2C12 myoblasts were cocultured with adult rat cardiomyocytes to evaluate whether signals generated by cardiomyocytes—by direct cell-cell contacts and/or by the release of soluble factors—could modulate the expression and function of myoblastic Cx43, thus favoring myoblast commitment to a more integrable cardiomyocyte-like phenotype. The effects of the hormone relaxin, which has cardiotropic actions (2) and binds to specific receptors in the heart (12, 22), in influencing the intercellular coupling between the two cell types was also investigated. This also was studied in consideration of the suggested role for this hormone in the modulation of the growth and/or differentiation of cardiomyocytes during fetal and neonatal life (31).

MATERIALS AND METHODS

Cell cultures and cocultures. Mouse skeletal C2C12 myoblasts (American Type Culture Collection, Manassas, VA) were cultured in DMEM containing 10% fetal bovine serum (Sigma, Milan, Italy) and 0.1% gentamicin and grown in a 5% CO2 atmosphere at 37°C. In some experiments, myoblasts were cultured in medium containing 0.5, 1, or 1.5 mM Ca2+ and used for Western blot analysis of Cx43 expression. Primary cultures of rat ventricular cardiomyocytes were prepared from the hearts of male adult Wistar rats (Harlan, Correzzana, Italy) by collagenase dispersion as previously described (20). On the day of isolation, cardiomyocytes and myoblasts were mixed at a 1:3 ratio in DMEM/medium 199 (M199) and plated onto laminin-coated coverslips. To study the effects of direct cell-to-cell contacts on myoblasts, cocultures were maintained in M199 supplemented with coated coverslips. To study the effects of direct cell-to-cell contacts on a 1:3 ratio in DMEM/medium 199 (M199) and plated onto laminin-zana, Italy) by collagenase dispersion as previously described (20). Prepared from the hearts of male adult Wistar rats (Harlan, Correzzana, Italy) by collagenase dispersion as previously described (20). To achieve this purpose, mouse C2C12 myoblasts were cocultured with adult rat cardiomyocytes to evaluate whether signals generated by cardiomyocytes—by direct cell-cell contacts and/or by the release of soluble factors—could modulate the expression and function of myoblastic Cx43, thus favoring myoblast commitment to a more integrable cardiomyocyte-like phenotype. The effects of the hormone relaxin, which has cardiotropic actions (2) and binds to specific receptors in the heart (12, 22), in influencing the intercellular coupling between the two cell types was also investigated. This also was studied in consideration of the suggested role for this hormone in the modulation of the growth and/or differentiation of cardiomyocytes during fetal and neonatal life (31).

Confocal immunofluorescence microscopy. To reveal Cx43 expression, paraformaldehyde-fixed myoblasts in monoculture and in coculture with cardiomyocytes were incubated with mouse monoclonal anti-Cx43 antibody (1:200 dilution; Chemicon, Temecula, CA) and Alexa-conjugated goat anti-mouse IgG (1:100 dilution; Molecular Probes, Eugene, OR). Counterstaining was performed with propidium iodide (Molecular Probes). Negative control experiments were performed by replacing the primary antibody with nonimmune mouse serum. Cells were examined with a Bio-Rad MCR 1024 ES confocal laser scanning microscope (Bio-Rad, Hercules, CA) equipped with a 15-mW Kr-Ar laser for fluorescence measurements and with differential interference contrast (DIC) optics for transmission images. Fluorescence was collected using a Nikon PlanApo ×60 oil-immersion lens objective. Series of optical sections (512 × 512 pixels) at intervals of 0.4 μm were obtained and superimposed to create a single composite image. The laser potency, photomultiplier, and pinhole size were kept constant. When needed, fluorescence and DIC images were merged to view the precise distribution of the immunostaining. Semi quantitative analysis of the intensity of the immunostaining for Cx43 expression was performed on digitized images of whole myoblasts or myoblast clusters using the Scion Image Beta 4.0.2 image analysis software program (Scion, Frederick, MD). To evaluate Cx43 expression in the gap junctions, the optical density of Cx43 immunoreactivity at the contact sites among adjacent myoblasts was also analyzed. At least 30 different cells were analyzed in each experimental group, and the mean ± SE optical density was then calculated. Statistical analysis of differences between the experimental groups was performed using one-way ANOVA and the Newman-Keuls posttest. Calculations were performed using the GraphPad Prism statistical software program (GraphPad, San Diego, CA).

Immunoprecipitation and Western blot analysis. For immunoprecipitation, cells were lysed for 15 min in lysis buffer containing (in mM) 20 Tris, pH 7.4, 150 NaCl, 1 EDTA, 1 EGTA, 1 PMSF, 1% Triton X-100, and protease inhibitor cocktail (Roche) sonicated on ice twice for 5 s each time and centrifuged for 15 min at 10,000 g. Supernatant was incubated with 5 μg of rabbit polyclonal anti-Cx43 antibody (Santa Cruz Biotechnology, Milan, Italy) on a rocking platform. After 2 h, 50 μl of protein G agarose beads (Sigma) were added and incubated for 2 h on a rocking platform. After precipitation, the protein G agarose-protein complex was washed with lysis buffer and analyzed using Western blotting. Protein samples were resolved using 8% PAGE and transferred onto a nitrocellulose membrane.
Nonspecific sites were blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20 (T-PBS), pH 7.8. The blot was immunostained with anti-Cx43 (1:200 dilution) and anti-rabbit-horseradish peroxidase conjugate (1:10,000 dilution; Santa Cruz Biotechnology) and developed using the Opti-4CN substrate kit (Bio-Rad).

Lucifer yellow dye transfer analyses. To reveal functional gap junctions, the gap junction-permeant dye Lucifer yellow (20% in PBS; Molecular Probes) was microinjected into single cells using a pressure injection system (Femtojet InjectMan NI2; Eppendorf, Hamburg, Germany) under a phase-contrast microscope. The fluorescent coupling was viewed under a Nikon Diaphot 300 microscope equipped with fluorescence illumination and FITC filters (excitation, 488 nm; emission, 512 nm) and photographed using a Nikon digital camera with which we obtained one image per second. The specificity of dye transfer was tested by pretreatment with 1 mM heptanol, a blocker of gap junction coupling. In the coculture experiments, myoblasts in close apposition with cardiomyocytes were microinjected with the dye. The extent of gap junction intercellular communication was quantified by counting the number of fluorescent cells surrounding the microinjected cells (number of dye-coupled cells per microinjection). At least 20 independent microinjections were performed for each sample.

Electrophysiology. Electrophysiological properties of gap junction channels between homologous myoblast-myoblast and heterologous myoblast-cardiomyocyte cell pairs were analyzed using dual whole cell patch clamping. Coverslips with adherent myoblasts in monolayer or in coculture with cardiomyocytes were placed, after 48 h of culture, onto the stage of a Nikon Eclipse TE 2000 inverted micro-

Fig. 2. Confocal immunofluorescence images showing Cx43 expression in the cocultures. Myoblasts were cultured in direct contact with cardiomyocytes or in the presence of cardiomyocyte conditioned medium for 48 h with or without relaxin. A: DIC image of cocultures showing cardiomyocytes (arrows) surrounded by myoblasts. B–D: superimposed fluorescence and DIC images of cocultures showing Cx43 immunoreactivity in the absence (B) and presence (C and D) of relaxin. Cx43 is highly expressed around the nuclei as well as at the junction (arrowheads in D) between the two cell types. Cardiomyocytes are indicated by white circles in B and C and labeled as cm in D. Representative images of at least 10 independent experiments with similar results are shown. A–C: integrated optical sections. D: single optical section. E: densitometric analysis of Cx43 immunofluorescence in whole myoblasts (open bars) or at the gap junctional appositions on the plasma membrane (hatched bars). In both cases, the levels of Cx43 are markedly increased in the cocultures, especially upon relaxin treatment. *P < 0.05 and **P < 0.001 vs. myoblasts alone and myoblasts + conditioned medium. ##P < 0.001 vs. other groups.
Fig. 3. Immunoprecipitation and Western blot analysis of myoblasts cultured in direct contact with cardiomyocytes or in the presence of cardiomyocyte conditioned medium for 48 h with or without relaxin. The levels of myoblast-specific Cx43 appear greatly increased in the cocultures and this effect is potentiated by relaxin. Images representative of 3 independent experiments with similar results are shown.

Fig. 4. Lucifer yellow dye/transfer analysis of gap junction communication. Superimposed phase-contrast and fluorescence images of myoblasts in monoculture (A), in coculture with cardiomyocytes (encircled by thin white lines in B and C) for 48 h (B), and in coculture after treatment with relaxin (C). Arrows indicate the microinjected cells. Representative images of 5 independent experiments with similar results are shown.
myocytes and myoblasts were cocultured on coverslips for 48 h and measurement of fluo-3 AM (Molecular Probes) fluorescence. Cardiac contractions frequently could be observed in A2). Spontaneous contractions typically occurred in the cocultures either in basal conditions, by choosing spontaneously beating cardiomyocytes, or after stimulation with caffeine (10 mM) or isoproterenol (25 nM), both of which are capable of selectively increasing intracellular Ca2+ in cardiomyocytes with no effects on myoblasts (8, 25). Fluorescence images were acquired using confocal microscopy with a 488-nm excitation wavelength and a 510-nm emission wavelength. Images (512 × 512 pixels) were acquired every 0.35 s. Time course analysis of Ca2+ transients was performed using Time Course software (Bio-Rad).

RESULTS

Expression of Cx43 by myoblasts. Under phase-contrast microscopy, myoblasts in monocultures appeared flattened, elongated, or spindle shaped with cytoplasmic projections anchoring the cells to the substrate. Confocal immunofluorescence, performed to reveal the expression and spatial distribution of Cx43 protein in myoblasts, showed that these cells had a small amount of Cx43 after 24 or 48 h in culture, with a few fluorescent dots concentrated mainly around the nuclei and on the plasma membrane at regions of contact with neighboring cells (Fig. 1). In coculture with cardiomyocytes, myoblasts retained their morphological features, while cardiomyocytes had a rodlike shape and appeared as single elements or small clusters firmly adhering to the surrounding myoblasts (Fig. 2A). Spontaneous contractions frequently could be observed in the cardiomyocytes at the time of isolation as well as after 24 h of coculture, with the tendency to reduce over time. Of interest, myoblasts in the cocultures showed increased Cx43 immunoreactivity compared with the same cells cultured alone. The inductive effect was greater after 48 h of coculture, with abundant Cx43 immunofluorescent dots in the perinuclear regions of myoblasts, at the cell borders between myoblasts, and at the myoblast-cardiomyocyte interface (Fig. 2B). In the long-term cocultures (72 and 96 h), the expression of Cx43 in myoblasts remained substantially unmodified compared with that found at 48 h (data not shown), suggesting that 48 h of coculture represents a turning point for the acquisition of the gap junction protein by these cells. Of interest, the induction of Cx43 expression was even more pronounced when the cocultures were treated with relaxin (100 ng/ml) for 48 h (Fig. 2C). In most cases, clear-cut, punctate, linear appositional staining was observed between myoblasts and cardiomyocytes (Fig. 2D). To investigate the mechanisms involved in the cardiomyocyte-induced upregulation of Cx43 expression in myoblasts, confocal immunofluorescence microscopic imaging was also performed in myoblasts separated from cardiomyocytes by polycarbonate membranes, enabling diffusion of soluble factors but preventing contact between cells, in the absence and in the presence (100 ng/ml) of relaxin. We found that cardiomyocyte-conditioned medium did not exert any substantial inductive effect on Cx43 expression by myoblasts, regardless of the addition of relaxin. Densitometric analysis of Cx43 immunostaining performed in all experimental conditions confirmed the visual findings (Fig. 2E). Of note, differentiation of myoblasts into myotubes, the direct skeletal muscle fiber precursors, was never observed in our experimental conditions.

Western blotting also showed that myoblasts cocultured in direct contact with cardiomyocytes, especially in the presence of relaxin (100 ng/ml), expressed higher levels of Cx43 compared with myoblasts in monoculture (Fig. 3). On the other hand, no substantial changes were observed in the myoblasts grown in cardiomyocyte-conditioned medium, with or without relaxin.

Gap junction functional assay using Lucifer yellow. The enhanced expression of Cx43 in the cocultured myoblasts also increased cell coupling, as shown by microinjection/dye-transfer assay with Lucifer yellow. The efficacy of dye spreading among myoblasts after 48 h of monolayer culture was very low, with ~50% of the cells showing dye coupling with only one or two neighboring cells (Fig. 4A). Moreover, dye spreading required 15–20 s. Conversely, the number of myoblasts recruited per microinjection, as well as the rate of dye spreading, was significantly enhanced after 48 h of coculture, reaching the highest values after treatment with relaxin (100 ng/ml), with 80% of the cells showing 5–6 and 10–12 coupled cells per injection, respectively, and a mean dye spreading time of 2–4 s (Fig. 4B). Of interest, Lucifer yellow also propagated from myoblasts to cardiomyocytes and vice versa (Fig. 4C), suggesting that the two cell types were physically connected through gap junctions. Inhibition of dye transfer by heptanol (1 mM) further proved the existence of functional gap junctions among myoblasts and between myoblasts and cardiomyocytes (data not shown).

<table>
<thead>
<tr>
<th>Culture</th>
<th>Relaxin</th>
<th>Gj, nS</th>
<th>relaxin heptanol, nS</th>
<th>No. of GJ studied</th>
<th>%GJ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoculture</td>
<td>Myoblasts</td>
<td>−</td>
<td>5.61±0.82</td>
<td>0.62±0.04 (6)</td>
<td>20</td>
</tr>
<tr>
<td>Myoblast</td>
<td>−</td>
<td>8.41±1.23</td>
<td>0.71±0.05 (5)</td>
<td>18</td>
<td>81.8</td>
</tr>
<tr>
<td>Cardiomyocyte</td>
<td>−</td>
<td>4.10±0.70*</td>
<td>0.61±0.06 (6)</td>
<td>14</td>
<td>63.6</td>
</tr>
<tr>
<td>Myoblast</td>
<td>+</td>
<td>15.11±2.12‡</td>
<td>0.71±0.04 (5)</td>
<td>14</td>
<td>93.3*</td>
</tr>
<tr>
<td>Cardiomyocyte</td>
<td>+</td>
<td>12.20±2.41§</td>
<td>0.63±0.05 (6)</td>
<td>13</td>
<td>92.8†</td>
</tr>
</tbody>
</table>

Trans junctional conductance, Gj, columns 4 and 5, measured at a Vj = 10 mV in monolayers of myoblasts and cocultures of myoblasts and cardiomyocytes. Voltage pulses were applied to a myoblast (cell 1), and Ij was measured from the other cell of the pair (cell 2), myoblast or cardiomyocyte, as indicated in column 2. In column 3 is indicated relaxin treatment (+). In column 5 are Gj values determined in some cell pairs after pretreatment with heptanol (number in brackets indicates total treated cell pairs). In column 6 is reported the number of gap junctions studied, and column 7 lists their percentage with respect to the total number of cell pairs investigated (%GJ). *P < 0.05 and ‡P < 0.01 for gap junctions in homologous myoblast-myoblast pairs in cocultures vs. monolayers, respectively. †P < 0.05 and §P < 0.01 for gap junctions in homologous myoblast-myoblast and heterologous myoblast-cardiomyocyte pairs in cocultures treated with relaxin vs. untreated cell pairs, respectively. All Gj values found after heptanol treatment (column 5) are significantly (P < 0.005) lower than those of column 4. Significant differences were evaluated using one-way ANOVA.
Gap junction functional assay by electrophysiology. Currents recorded from cell 2 (myoblasts or cardiomyocyte) generated using voltage steps of +10 mV ($I_J$-negative inward current) and $-10$ mV ($I_J$-positive outward current) applied to cell 1 (myoblast) had a similar amplitude. Table 1 summarizes gap junctional conductance at a transjunctional voltage ($V_J$) of +10 mV for all cell pairs studied. Transjunctional conductance $G_J$ estimated at +10 mV from homologous myoblast-myoblast cell pairs, was significantly smaller in monocultures than in cocultures ($\sim 5.6$ vs. 8.4 nS). Moreover, $G_J$ estimated from heterologous myoblast-cardiomyocyte cell pairs was significantly less ($\sim 4.0$ nS) than that of myoblast-myoblast pairs in cocultures. When the cocultures were treated with relaxin, $G_J$ increased significantly in both myoblast-myoblast ($\sim 15.1$ nS) and myoblast-cardiomyocyte ($\sim 12.2$ nS) pairs. The percentage of functional gap junctions between homologous myoblast-myoblast pairs increased significantly in coculture compared with that in monoculture (Table 1). Moreover, treatment with relaxin caused a further increase in the percentage of functional coupling between the homologous and heterologous cell pairs.
(Table 1). The relationship between \( V_j \) and \( I_j \) was studied in myoblast-myoblast pairs in monocultures and cocultures, as well as in myoblast-cardiomyocyte pairs. Figure 5 shows representative current traces \( (I_j) \) of all cell pairs studied. The electrical coupling between myoblast-myoblast and myoblast-cardiomyocyte pairs was characterized by quasisymmetrical \( I_j \) (Fig. 5, A and C) and asymmetrical \( I_j \) (Fig. 5D), respectively. In particular, \( I_j \) from homologous cell pairs in coculture was larger in amplitude than that in monoculture (Fig. 5, A and C) and was almost twofold that of heterologous cell pairs (Fig. 5, C and D). After relaxin treatment, \( I_j \) in both cell pairs doubled in amplitude (Fig. 5, C-F). Heptanol (1 mM) strongly decreased both \( I_j \) and \( G_j \) within 3–5 min, because \( G_j \) at +10 mV ranged from 0.62 to 0.72 nS (Table 1) in all cell pairs investigated (Fig. 5B), suggesting that the measured conductance was dependent on functional gap junctional channels rather than on alternative modes of current flow. The conductances \( G_{j,\text{inst}} \) and \( G_{j,\text{ss}} \) were also estimated, and their values were normalized and plotted against \( V_j \) (Fig. 6). \( G_{j,\text{inst}} \) was virtually independent of the voltage (Fig. 6, open symbols), whereas \( G_{j,\text{ss}} \) showed a voltage dependence with a quasisymmetric or asymmetric pattern (Fig. 6, closed symbols). In particular, myoblast-myoblast pairs in monocultures and cocultures showed a quasisymmetric \( G_{j,\text{ss}} \) vs. \( V_j \) plot (Fig. 6, A and B), because \( V_j \) was significantly larger and \( G_{\text{min}} \) was smaller at positive \( V_j \) values than at negative \( V_j \) values (Table 2), and 2) the voltage sensitivity (the \( A \) constant in the Boltzmann function) at negative and positive \( V_j \) values was not statistically different. These data indicate the presence of a dominant connexin isoform to form the intercellular channels between homologous cell pairs in both the monoculture and the coculture. Conversely, in myoblast-cardiomyocyte pairs, the \( G_{j,\text{ss}} \) vs. \( V_j \) plots were clearly asymmetric (Fig. 6C), suggesting the presence of different connexin isoforms. This asymmetrical behavior determined a constant inward current toward the paired cardiomyocyte with negative \( V_j \) value. In heterologous cell pairs, the \( A \) and \( V_0 \) Boltzmann parameters at positive \( V_j \) values were significantly different from those of homologous cell pairs. Relaxin did not change the voltage dependence of the normalized \( G_{j,\text{ss}} \) in either homologous or heterologous cell pairs at positive \( V_j \) values (Fig. 6, B and C, closed triangles; Table 3), whereas it modified this pattern at negative \( V_j \) values in homologous cell pairs (Fig. 6B, closed triangles). Indeed, in this latter condition, \( G_{j,\text{ss}} \) vs. \( V_j \) plots were asymmetrical, producing a constant inward current toward the paired myoblast with negative \( V_j \) value.

**Intercellular propagation of Ca\(^{2+}\) waves.** Because gap junctions play a fundamental role in mediating intercellular signaling and coordinating cellular behavior, we next examined whether gap junctions were involved in the transmission of intercellular Ca\(^{2+}\) signals between cardiomyocytes and myoblasts upon loading with the Ca\(^{2+}\)-sensitive dye fluo-3 AM. After 48 h of coculture, spontaneous Ca\(^{2+}\) oscillations could be observed in some of the cardiomyocytes, which propagated toward the surrounding myoblastic cells. This phenomenon was even more pronounced after stimulation with pharmacological agents such as isoproterenol and caffeine to induce a large and sustained Ca\(^{2+}\) rise in the cardiomyocytes (Fig. 7). Ion coupling between the two cell types was prevented by pretreatment with 1 mM heptanol (data not shown), indicating gap junction-mediated intercellular Ca\(^{2+}\) waves.

**Role of Ca\(^{2+}\) in the upregulation of Cx43 expression.** Because cytosolic Ca\(^{2+}\) plays a critical role in the cascade of events involved in myoblast differentiation, we next wondered whether Ca\(^{2+}\) waves propagating from contracting cardiomyocytes could influence the upregulation of the cardiac connexin isoform Cx43. Indeed, in heterologous myoblast-cardiomyocyte pairs, the \( G_{j,\text{ss}} \) values were significantly different from those of homologous cell pairs at positive \( V_j \) (Fig. 6D), whereas it modified this pattern at negative \( V_j \) (Fig. 6C), and asymmetrical for heterologous cell pairs (Fig. 6B, closed triangles). The continuous curves represent the best fit of the Boltzmann function to \( G_{j,\text{ss}} \).
FUNCTIONAL INTERACTIONS BETWEEN MYOBLASTS AND CARDIOMYOCYTES

Table 2. Boltzmann parameters of the normalized transjunctional conductance for gap junctions in cell pairs

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Monoculture</th>
<th>Homologous pairs</th>
<th>Heterologous pairs</th>
<th>Coculture</th>
<th>Homologous pairs</th>
<th>Heterologous pairs</th>
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<tbody>
<tr>
<td></td>
<td>−V&lt;sub&gt;j&lt;/sub&gt;</td>
<td>+V&lt;sub&gt;j&lt;/sub&gt;</td>
<td>−V&lt;sub&gt;j&lt;/sub&gt;</td>
<td>+V&lt;sub&gt;j&lt;/sub&gt;</td>
<td>−V&lt;sub&gt;j&lt;/sub&gt;</td>
<td>+V&lt;sub&gt;j&lt;/sub&gt;</td>
</tr>
<tr>
<td>A&lt;sub&gt;0&lt;/sub&gt;, mV&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>0.06 ± 0.003</td>
<td>0.05 ± 0.005</td>
<td>0.06 ± 0.004†</td>
<td>0.064 ± 0.005†</td>
<td>0.053 ± 0.004‡</td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;c&lt;/sub&gt;, mV</td>
<td>−51.11 ± 6.32</td>
<td>82.10 ± 5.42*</td>
<td>−67.02 ± 6.02</td>
<td>74.03 ± 6.23*†</td>
<td>82.07 ± 7.02‡</td>
<td></td>
</tr>
<tr>
<td>G&lt;sub&gt;min&lt;/sub&gt;</td>
<td>0.48 ± 0.03</td>
<td>0.30 ± 0.02*</td>
<td>0.51 ± 0.14</td>
<td>0.40 ± 0.05*†</td>
<td>0.39 ± 0.04§</td>
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<td>G&lt;sub&gt;max&lt;/sub&gt;</td>
<td>1.02 ± 0.02</td>
<td>1.01 ± 0.04</td>
<td>1.00 ± 0.04</td>
<td>1.01 ± 0.06</td>
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Parameters obtained by fitting the Boltzmann function on the normalized steady-state conductance (G<sub>j</sub>,<sub>0</sub>) vs. negative or positive V<sub>j</sub> (−V<sub>j</sub> and +V<sub>j</sub>, respectively) from 20 homologous myoblast-myoblast pairs in monocultures (columns 2 and 3) and 18 homologous (columns 4 and 5) and 14 heterologous myoblast-cardiomyocyte (columns 6 and 7) pairs in coculture. Quasisymmetrical G<sub>j</sub> inactivation is observed in myoblast pairs in both monocultures and cocultures, whereas an asymmetrical G<sub>j</sub> is observed in myoblast-cardiomyocyte pairs. In the latter pairs, G<sub>j</sub> at negative V<sub>j</sub> remains constant over time (column 6). Data are presented as means ± SE. G<sub>max</sub>, maximum conductance; G<sub>min</sub>, voltage-insensitive residual conductance; A, voltage sensitivity; V<sub>c</sub>, transjunctional voltage halfway between G<sub>max</sub> and G<sub>min</sub>. *P < 0.05 for +V<sub>j</sub> vs. −V<sub>j</sub> of the same pairs. †P < 0.05 for +V<sub>j</sub> vs. −V<sub>j</sub> of the same pairs. ‡P < 0.05 for +V<sub>j</sub> of heterologous myoblast-cardiomyocyte vs. homologous myoblast-myoblast pairs in coculture. §P < 0.05 for myoblast-cardiomyocyte pairs vs. homologous cell pairs in cocultures. Significant differences were evaluated using one-way ANOVA.

DISCUSSION

The basic understanding of the mechanisms involved in the intercellular communication between cardiomyocytes and skeletal myoblasts may have crucial importance in refining treatment techniques and improving functional outcomes for patients with CCM. Unfortunately, only little effort has been made in this direction; most of the studies performed to date have reported conflicting results regarding the efficiency of myoblast grafting in the postischemic heart (11, 15, 19, 25, 26, 30). In the present study, we have demonstrated that myoblasts establish mechanical and electrical coupling with adjacent cardiomyocytes in an in vitro coculture model. Moreover, the expression of Cx43, the most common connexin isoform of gap junction channels, was increased in the myoblasts upon coculture with cardiomyocytes, thus providing novel experimental evidence in favor of a role for cardiomyocytes in the control of intercellular communication among myoblasts. The close correlation found between Cx43 expression and the extent of Lucifer yellow dye spreading among neighboring myoblasts strongly suggests that cardiomyocyte-induced facilitation of intercellular coupling is mediated by an enhancement in the number and function of gap junctions. Direct cell-to-cell contacts between the two cell types appear to be necessary for myoblasts to upregulate Cx43 expression, because conditioned medium containing soluble factors released from cardiomyocytes caused only a slight increase in the myoblastic levels of Cx43 compared with those obtained in the monocultures. This finding suggests that juxtacrine signaling from cardiomyocytes may be more involved than paracrine signaling in the regulation of Cx43 expression by myoblasts. The addition of the cardiotropic hormone relaxin to the cocultures potentiated the inductive effect of cardiomyocytes on myoblastic gap junctions. The above results were further confirmed and extended by analyzing the electrical properties of gap junctions in the cell pairs. This analysis showed that I<sub>j</sub> and G<sub>j</sub> between myoblast pairs increased markedly in the cocultures, especially in the presence of relaxin. Of interest, relaxin also was able to cause an approximately twofold increase in the electrical coupling between heterologous myoblast-cardiomyocyte cell pairs, suggesting that a more efficient intercellular exchange of signals between coupled cells occurred after treatment with relaxin. This novel cardiac effect of relaxin is consistent with previous studies showing that 1) cardiomyocytes express specific relaxin receptors (12, 22), the heart produces and releases intrinsic relaxin (7, 31), and 3) relaxin may play a role in modulating the growth and/or differentiation of cardiomyocytes during fetal and neonatal life (31).

There are at least two possible explanations for the ability of relaxin to affect intercellular coupling between homologous and heterologous cell pairs. The first and most obvious one relies on the increased Cx43 expression and gap junction channel formation observed in myoblasts cocultured with cardiomyocytes in the presence of relaxin. The second one might involve a more direct effect of relaxin on the preexisting channels. In fact, there is evidence that gap junction conductance can be regulated by phosphorylation via protein kinase-operated signal transduction pathways (5), including those activated by the relaxin receptor (12). A clue to the latter mechanism comes from our electrophysiological findings showing that the voltage dependence of I<sub>j</sub> between myoblast pairs in coculture changed from quasisymmetric to asymmetric after relaxin treatment, thus generating an inward current to the cell at lower membrane potential, with only marginal inactivation. It is conceivable that the sustained current flow between

Table 3. Boltzmann parameters for normalized transjunctional conductance in cell pairs in cocultures with relaxin

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Homologous, +V&lt;sub&gt;j&lt;/sub&gt;</th>
<th>Heterologous, +V&lt;sub&gt;j&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>A&lt;sub&gt;0&lt;/sub&gt;, mV&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>0.06 ± 0.003</td>
<td>0.05 ± 0.005</td>
</tr>
<tr>
<td>V&lt;sub&gt;c&lt;/sub&gt;, mV</td>
<td>80.03 ± 8.7</td>
<td>78.04 ± 8.6</td>
</tr>
<tr>
<td>G&lt;sub&gt;min&lt;/sub&gt;</td>
<td>0.39 ± 0.04</td>
<td>0.38 ± 0.03</td>
</tr>
<tr>
<td>G&lt;sub&gt;max&lt;/sub&gt;</td>
<td>1.01 ± 0.02</td>
<td>1.01 ± 0.01</td>
</tr>
</tbody>
</table>

Parameters obtained by fitting the normalized steady-state conductance (G<sub>j</sub>,<sub>0</sub>) vs. positive V<sub>j</sub> (+V<sub>j</sub>) from 14 myoblast-myoblast pairs and 10 myoblast-cardiomyocyte pairs with a Boltzmann function. Data are presented as means ± SE. No data evaluated using one-way ANOVA test were statistically different.

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myoblasts with different membrane potentials may also contribute to the explanation for the increased Lucifer yellow spreading among myoblasts after relaxin treatment. An asymmetrical voltage dependence of $I_J$ also characterized the heterologous myoblast-cardiomyocyte pairs and was likely dependent on species-related differences in Cx43 proteins and/or on the presence of multiple connexin isoforms in the heart (33). Of note, cardiomyocytes have a lower resting membrane potential than myoblasts ($-80$ vs. $-20$ mV, respectively) and undergo rhythmic changes of membrane potential. Therefore, the asymmetry of heterologous gap junctions may allow myoblasts to influence the resting potential and favor action potential generation in the coupled cardiomyocytes, thereby increasing their beating frequency, especially in the presence of relaxin.

Gap junctions coordinate many cellular activities by controlling the exchange of small regulatory mediators between adjacent cells, including Ca$^{2+}$ (3, 16). In our model, spontaneously beating cardiomyocytes could efficiently transfer Ca$^{2+}$ to the adjacent myoblasts via gap junctional coupling, and the expression of Cx43 by myoblasts was upregulated by Ca$^{2+}$ as also found in other cell types (14). All of these data suggest that the establishment of gap junctions between myoblasts and cardiomyocytes may trigger Ca$^{2+}$-dependent mechanisms leading to the increase in Cx43 expression that, in turn, reinforces intercellular coupling between the two cell types. Because Ca$^{2+}$ signaling is known to regulate cell differentiation (3), it is tempting to speculate that increased Ca$^{2+}$ propagation by functional gap junctions from spontaneously beating cardiomyocytes may modulate the differentiation pattern of myoblasts and possibly may facilitate their transdifferentiation toward a cardiac-like phenotype as previously suggested (13).

In conclusion, our study offers evidence that 1) undifferentiated skeletal myoblasts can establish functional gap junctions with cardiomyocytes, 2) direct cell-to-cell contacts are required to improve functional coupling between myoblasts and cardiomyocytes, and 3) the cardiotropic hormone relaxin potentiates...
the exchange of signals among coupled cells. We are aware that the results of in vitro studies cannot be applied directly in vivo, and experiments to test the integration of grafted myoblasts with the rat and pig myocardium in vivo are ongoing in our laboratory. However, the present findings may provide further support for using skeletal myoblasts as a promising tool for the repair of postinfarcted myocardium and for considering relaxin as a potential adjuvant for CCM. This latter possibility arises from previous studies showing that this hormone is able to increase perfusion in the injured cardiac tissue (17), induce collagen remodeling (2, 7), counteract cardiac fibrosis (27), and produce neangiogenesis selectively in tissue areas under repair (32), suggesting that relaxin might also be useful in CCM by reducing scar formation and increasing the microvesSEL network in the damaged myocardium.

REFERENCES