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Connexin expression in cultured neonatal rat myocytes reflects the pattern of the intact ventricle

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Abstract

Objective: Primary cultures of neonatal rat ventricular myocytes have become a widely used model to examine a variety of functional, physiological and biochemical cardiac properties. In the adult rat, connexin43 (Cx43) is the major gap junction protein present in the working myocardium. In situ hybridization studies on developing rats, however, showed that Cx40 mRNA displays a dynamic and heterogeneous pattern of expression in the ventricular myocardium around birth. The present studies were performed to examine the expression pattern of the Cx40 protein in neonatal rat heart, and to examine the connexins present in cultures of ventricular myocytes obtained from those hearts. **Methods**: Cryosections were made of hearts of 1-day-old Wistar rats. Cultures of ventricular myocytes obtained from these hearts by enzymatic dissociation were seeded at various densities (to obtain >75, ~50%, and <25% confluency) and cultured for 24, 48 or 96 h. Cx40 and Cx43 were detected by immunofluorescence and immunoblotting. **Results:** Immunohistochemical stainings confirmed that gap junctions in the atrium and His–Purkinje system were composed of at least Cx43 and Cx40. From the subendocardium towards the subepicardium Cx40 expression gradually decreased, resulting in the sole expression of Cx43 in the subepicardial part of the ventricular wall. In ventricular myocytes cultured at high density (>75% confluency) Cx43 and Cx40 immunoreactivity could be detected. In contrast to Cx43 immunolabeling which showed a homogeneous distribution pattern, Cx40 staining was heterogeneous, i.e. in some clusters of cells abundant labeling was present whereas in others no Cx40 staining could be detected. The pattern of Cx43 immunoreactivity was not altered by the culture density. In contrast, in isolated ventricular myocytes cultured at low density (<25% confluency) the relative number of cell–cell interfaces that were Cx40-immunopositive decreased as compared to high density cultures (35 vs. 70%). Western blots did not reveal significant differences in the level of Cx40 and Cx43 expression at different culture densities. **Conclusions:** These results show that cultured ventricular myocytes retained typical features of the native neonatal rat ventricular myocardium with regard to their composition of gap junctions. This implicates that these cultures may serve as a good model for studying short-term and long-term regulation of cardiac gap junction channel expression and function.

Keywords: Cell culture/isolation; Developmental biology; Gap junctions; Histo(patho)logy; Myocytes

1. Introduction

Cardiac gap junction channels are essential for the successful propagation of electrical activity throughout the heart [1]. Gap junction channels are formed by connexin proteins, which are coded for by a multigene family consisting of at least 14 members in mammals (for reviews, see Bruzzone et al. [2], Gros and Jongsma [3] and Willecke and Haubrich [4]). Six connexin protein subunits form a hemichannel (or connexon) in the plasma membrane of one cell that can dock to its counterpart in the membrane of contacting cells (for reviews, see Kumar and Gilula [5] and Sosinsky [6]). Expression of several con-

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nexins by stable transfection of communication-incompetent cell lines have revealed that different connexins form channels with distinct conductance, permeability and regulatory properties [7–9].

Four different gap junction proteins, connexin43 (Cx43), Cx40, Cx45, and Cx37, have been identified to be expressed in the adult mammalian myocardium [10–12]. In addition to these four connexins, Cx46 mRNA has been detected in homogenates of mammalian hearts [13]. Although occasionally detected between human atrial and ventricular myocytes [14], Cx46 is considered to be attributable to valvular tissues in the rat heart [15]. Cx43 is generally regarded to be the most abundant cardiac connexin. It can be readily observed in gap junctions in the atrial and ventricular working myocardium and in the distal His–Purkinje system [16–20]. The distribution of Cx40 in the adult heart is more restricted. Cx40 is most abundantly present in the atrium and in the conduction system [17,18,21–24]. Cx45 has been reported to be expressed in myocytes of the conduction system as well as in the atrium and ventricle of dog, rat and human hearts [20,25,26]. Recently it has been shown, however, that Cx45 expression is confined to the ventricular conduction system only [27]. Finally, Cx37 is expressed in the endocardial layer of atria and ventricles [12,28].

The role of the different connexins expressed in heart is best illustrated in studies on cardiac conduction in connexin knock-out mice. Homozygous Cx43 $^{-}/^{-}$ mice die shortly after birth because of an obstruction of the right ventricular outflow tract [29,30]. However, studies on heterozygous Cx43 $^{+}/^{-}$ mice, expressing about half the normal level of Cx43 protein, indicated that Cx43 is the main determinant of conduction in the ventricular, but not in the atrial myocardium [31]. Studies of homozygous Cx40 $^{-}/^{-}$ mice, on the other hand, demonstrated the importance of this Cx for atrial and His–Purkinje conduction [32,33].

In the rat heart, the expression of Cx40 mRNA is developmentally regulated [34]. A strong expression of Cx40 mRNA in both atrial and ventricular myocytes of young embryos was shown using the in situ hybridisation technique. As development proceeds, the expression of Cx40 mRNA in the atria gradually extinguishes and is no longer detectable in the adult rat. The developing ventricles, on the other hand, show a more dynamic and heterogenous expression of Cx40 mRNA. The mRNA disappears progressively from the ventricular working myocardium, being just after birth still expressed in a subendocardial layer in the ventricles. In the adult rat, Cx40 mRNA is confined to the conduction system. With the exception of the atria, where Cx40 persists in adult life, for the developing mouse heart a comparable pattern of expression has been described [23].

Primary cultures of neonatal rat cardiac myocytes have become a widely used model to examine a variety of functional, physiological and biochemical cardiac properties. However, several laboratories use different isolation procedures and culture conditions, which might affect the basic and regulatory properties of the ventricular cells. In fact, it has been shown that after collagenase isolation neonatal rat cardiomyocytes restore their typical heart cell morphology after 2 days whereas after trypsine isolation usually a week is needed to reach a similar appearance [35]. It has also been reported that the expression of some cardiac genes, like SERCA2 and Cx43, might vary with the density of the plated cells and the amount of time in culture [36–38]. As a result a similar cultures prepared in different laboratories may display different characteristics due to differential expression of certain cardiac genes which might not always reflect the actual situation in the heart.

The objectives of the present study were two-fold. First, we wanted to determine the distribution of Cx40 in the neonatal rat ventricular myocardium at the protein level. In addition, we have examined the distribution pattern of Cx43 and Cx40 in cultures of isolated neonatal rat ventricular myocytes seeded at different densities and cultured for different length of time. The results presented in this study show that in sections of ventricle as well as in isolated ventricular myocytes both Cx43 and Cx40 immunoreactivity could be detected although in different distribution profiles. Furthermore, they show that our cultured neonatal rat ventricular myocytes retained typical features of the native neonatal rat ventricular myocardium with regard to their composition of gap junctions.

2. Methods

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1. Isolation and culture of neonatal rat ventricular cardiomyocytes

Primary cultures of neonatal rat ventricular cardiomyocytes were obtained from 1-day-old Wistar rats as described previously [39]. Briefly, the hearts were excised aseptically and atrial tissue and large vessels were trimmed off. Ventricles were cut into small pieces of about 1 mm^3, and incubated in 0.75 ml dissociation medium per heart. The dissociation medium was a HEPES-buffered physiological saline containing 450 U/ml collagenase (Worthington, CLS type 1), 0.001% DNAse (Worthington), 0.01 mM Ca^{2+} and 0.6 mM Mg^{2+}. Dissociation was obtained by gently stirring at 37°C. After 10 min the dissociation medium was discarded and replaced by a fresh aliquot. A further 40 min of stirring resulted in complete dissociation of the remaining fragments. The cell suspension was then cooled to 0°C for 5 min to inhibit enzyme activity. After

centrifugation, the cell pellet was washed and suspended in Ham's F10 culture medium (GIBCO) supplemented with 5% fetal calf serum and 5% horse serum (GIBCO). The fibroblast content of the cell suspension was reduced using a differential attachment method [40]. The cell suspension was transferred to 60-mm plastic culture dishes (Falcon 3004) and was kept for 90 min in an incubator at 37°C. After this preincubation, the myocytes remaining in suspension were counted with a hemocytometer and diluted to achieve 3×10^5 cells/ml. Aliquots of the cell suspension were plated at three different densities on glass coverslips or into plastic culture dishes (Falcon 3001). For high density cultures (>75% confluency), 1 ml of cell suspension was used per 35-mm culture dish. Consequently, 0.6 and 0.3 ml of the cell suspension was used to obtain medium density (~50% confluency) and low density (<25% confluency) cultures. These cultures, consisting predominantly of non-dividing ventricular myocytes, were incubated for 24, 48 or 96 h in a 5% CO_2/95% air- and humidity-controlled incubator at 37°C. Culture medium was refreshed each 24 h.

2.2. Antibodies

Polyclonal antibodies were raised in rabbit against oligopeptides of the carboxy-terminus of the gap junction proteins Cx40 (amino acids 335–356 [22]), and Cx43 (amino acids 314–322 [41]). A mouse monoclonal antibody raised against another part of the gap junction protein Cx43 (amino acids 252–270) was purchased from Zymed Laboratories Inc. To delineate the myocardium, a mouse monoclonal antibody against human desmin was used (Monosan). A mouse monoclonal antibody raised against Von Willebrandt factor (vWf) was used as a marker for endothelial cells (Dako).

2.3. Immunofluorescent labeling of cultured ventricular myocytes

Cells cultured on glass coverslips at three different densities (<25, ~50, and >75% confluency) for 24, 48 or 96 h were fixed in methanol at −20°C for 5 min. After fixation, cells were rinsed and incubated in 0.2% Triton X-100 in PBS for 1 h and subsequently incubated in 0.5 M NH_4Cl in PBS for 15 min. Cells were preincubated with 2% bovine serum albumin (BSA, Amersham) in PBS for 30 min and incubated overnight with primary antibody at appropriate dilutions (for mouse monoclonal anti-Cx43 1:1000, for affinity-purified rabbit polyclonal anti-Cx43 1–3 μg/ml, for anti-Cx40 3–5 μg/ml, for anti-desmin 1:50, and for anti-vWf 1:200) and 10% normal donkey serum (Jackson) in PBS. In between incubation steps cells were rinsed in PBS. After this period, the coverslips were rinsed three times for 5 min with PBS and incubated with secondary antibody (Texas red-conjugated donkey-anti-rabbit IgG at 1:100 or FITC-conjugated donkey-anti-mouse IgG at 1:100; Jackson) for 4 h followed by rinsing steps as described above. All steps were performed at room temperature. Coverslips were mounted on slides in Vectashield (Vector Laboratories) to reduce photobleaching. Cells were examined with a Nikon epifluorescence microscope equipped with appropriate filters.

2.4. Immunofluorescent labeling of heart cryosections

Immunofluorescent studies on cardiac tissue were performed as previously described [22]. Briefly, whole hearts were infiltrated with Tissue Tek OCT compound (Miles Laboratories) and rapidly frozen in liquid nitrogen. Serial sections (10 μm) were cut on a cryostat, collected on 3-aminopropyltriethoxysilane-coated slides and stored at −80°C until use. After equilibration to room temperature, sections were rehydrated in PBS and subjected to the same procedure as described above for immunofluorescent labeling of cultured ventricular myocytes.

2.5. Western blotting of cultured ventricular myocytes

Western blotting was performed as previously described [23]. Briefly, cells cultured at three different densities (<25, ~50, and >75% confluency) for 48 h were rinsed with cold PBS and subsequently scraped in ice-cold RIPA solubilization buffer consisting of 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 1% Nodinet-P40, 0.1% sodium dodecylsulfate, 2.5 mM pretreated sodium orthovanadate, 125 μM phenylarsine oxide and 2 mM phenylmethyl sulphonyl fluoride. Samples were maintained at −80°C, thereafter thawed and centrifuged for 30 min at $13\,000\times g$ at 4°C. Supernatants containing solubilized material were recovered and the total amounts of protein were quantified using a bicinchoninic acid quantification assay (Sigma). Fifty μg of protein was loaded on 12.5% SDS-polyacrylamide gel, electrophoresed and electrotransferred onto Nitroscreen membrane (Amersham). Membranes were then soaked for 1 h at room temperature in a 4% defatted milk saturation buffer consisting of 40 mM Tris–HCl (pH 7.5) and 0.1% Tween 20. Blotted proteins were probed for the presence of Cx40 or Cx43 by an overnight incubation at 4°C with rabbit polyclonal Cx40 (1 μg/ml; [22]) or Cx43 (1 μg/ml; [41]) antibodies. Complexed antigen-antibodies were detected with a BM chemiluminescence detection Western blotting reagent kit according to the manufacturer's instructions (Boehringer Mannheim). The chemiluminescence reaction was visualized on Hyperfilm MP (Amersham). Specificity of the labeling was checked by replacement of the antibody by an identical antibody preincubated overnight a 4°C with its immunogenic peptide (10 μg/ml).

3. Results

The distribution patterns of Cx43 and Cx40 in the heart of a 1-day-old rat were investigated in serial sections by immunofluorescence labeling. Fig. 1A shows the expression of the myocardial marker desmin in the left atrium (upper right corner) and the left ventricle (lower left corner). As expected Cx43 (Fig. 1E) is abundantly present in both atrial and ventricular myocytes, whereas the expression of Cx40 is restricted to the atrium (Fig. 1C). Fig. 1B shows the expression of desmin in a part of the ventricular myocardium, where some subendocardial Purkinje fibers are located on the right side of the picture whereas the left side of the picture shows the myocardium of the ventricular free wall adjacent to the subendocardium. Cx43 (Fig. 1F) can be detected in the Purkinje fibers and throughout the whole ventricular wall. However, Cx40 is abundantly labeled in both the Purkinje fibers and the subendocardial myocardium but staining intensity gradually decreases towards the outer layer of the ventricular free wall (Fig. 1D; left side of picture). Double immunostaining with mouse anti-Cx43 and rabbit anti-Cx40 antibodies examined under high magnification revealed colocalization of Cx40 with Cx43. However, whereas in the 'transition region' all cardiomyocytes showed a homogeneous Cx43 expression pattern, clusters of cardiomyocytes virtually

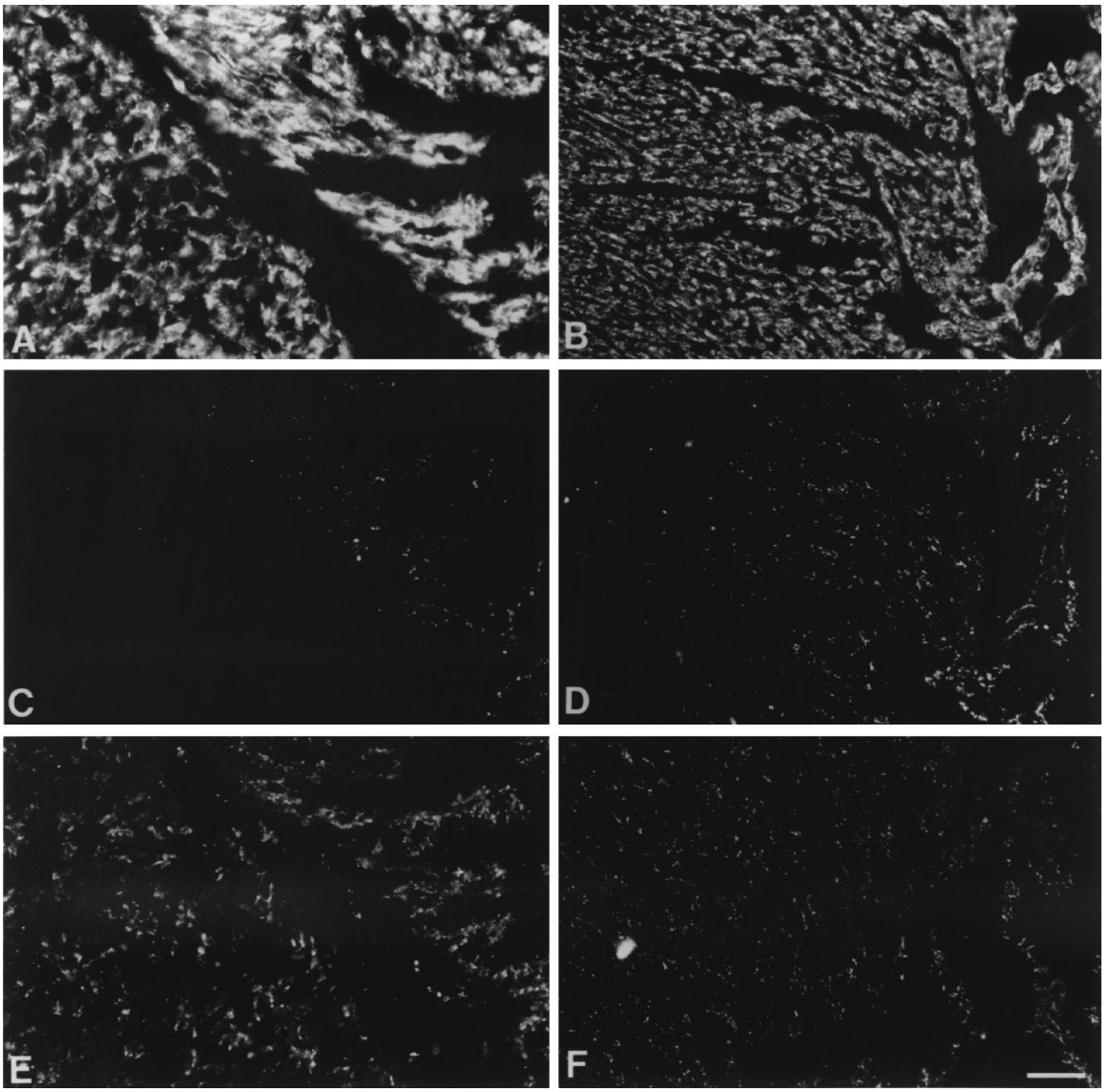

Fig. 1. Distribution of Cx40 and Cx43 in the neonatal rat heart. Immunofluorescence images of frozen sections (10 μm) of a 1-day-old rat are shown. Panels A, C and E (left side) and panels B, D and F (right side) display two groups of three adjacent sections incubated with antibodies against desmin, Cx40 and Cx43, respectively. Sections at the left side show the left atrium and the upper part of the left ventricular wall. Sections at the right side show part of the ventricular myocardium with some subendocardial Purkinje fibers. Note the gradual decline of Cx40 in D going from the endocardium (right) to epicardium (left). Bar represent 25 μm for panels A, C and E, and 50 μM for panels B, D and F.

devoid of Cx40 immunoreactivity were located next to clusters showing abundant Cx40 immunoreactivity (data not shown).

Immunofluorescent staining of ventricular myocytes was performed on cultures obtained by enzymatic dissociation of 1-day-old rat hearts, seeded at different densities and cultured for 24, 48 or 96 h. All anti-Cx antibodies produced identical staining patterns whatever the time of culture and therefore only the 48 h cultures will be discussed. Fig. 2A,B show a representative field from ventricular myocytes seeded at >75% confluency and double-labeled with mouse anti-Cx43 (panel A) and rabbit anti-Cx40 (panel B) antibodies. The anti-Cx43 antibody produced punctate labeling at appositional membranes between all cells, whereas the anti-Cx40 antibody labeled only between a few cells within a cluster. The colocalization of Cx40 with Cx43 at most of its immunoreactive sites is illustrated in Fig. 3, where the anti-Cx43 antibody is stained with FITC (green labeling) and the Cx40 antibody with Texas Red (red labeling), resulting in yellow labeling at gap junctional plaques containing both types of Cxs. Only sparse red labeling, indicating Cx40 immunoreactivity solely at a particular site, was observed throughout the preparation. Almost no intracellular labeling was observed with both antibodies. Control experiments using secondary antibodies alone yielded no significant labeling.

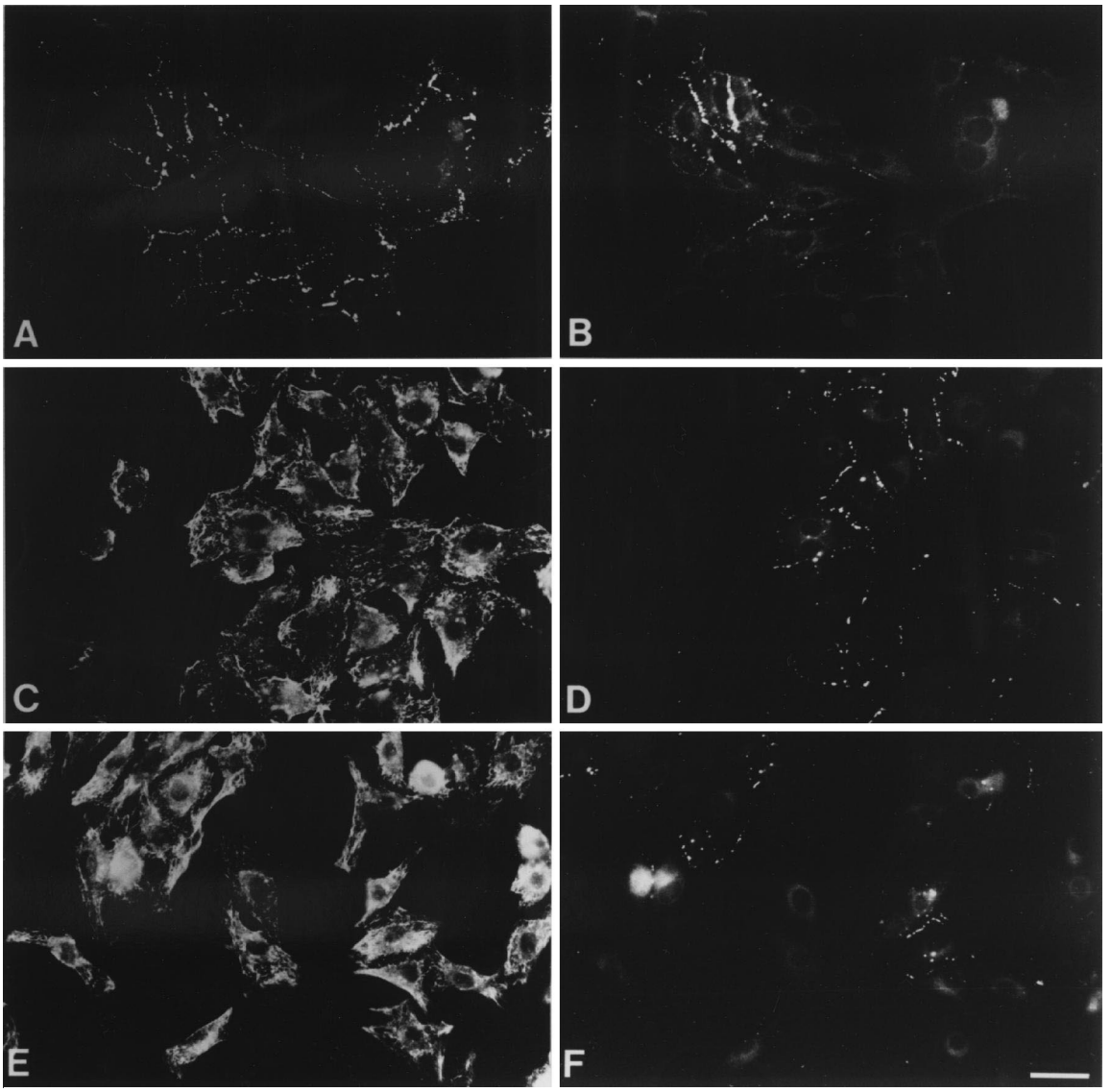

Fig. 2. Distribution of Cx40 in cultured neonatal rat ventricular myocytes varies with density. Panels A and B are a set of fluorescence images of neonatal rat ventricular myocytes cultured for 48 h at >75% confluency double-labeled with mouse anti-Cx43 (A) and rabbit anti-Cx40 (B). Whereas anti-Cx43 antibodies label virtually all cell–cell interfaces, the anti-Cx40 antibody labels only a few interfaces in a cluster of myocytes. Panels C and D and panels E and F are sets of fluorescence images of neonatal rat ventricular myocytes cultured for 48 h at >75% confluency and at ~50% confluency, respectively, double-labeled with mouse anti-desmin (C and E) and rabbit anti-Cx40 (D and F). Note the decrease in number of anti-Cx40 stained cell–cell interfaces going from >75 to ~50% confluency. Bar represents 25 μm.

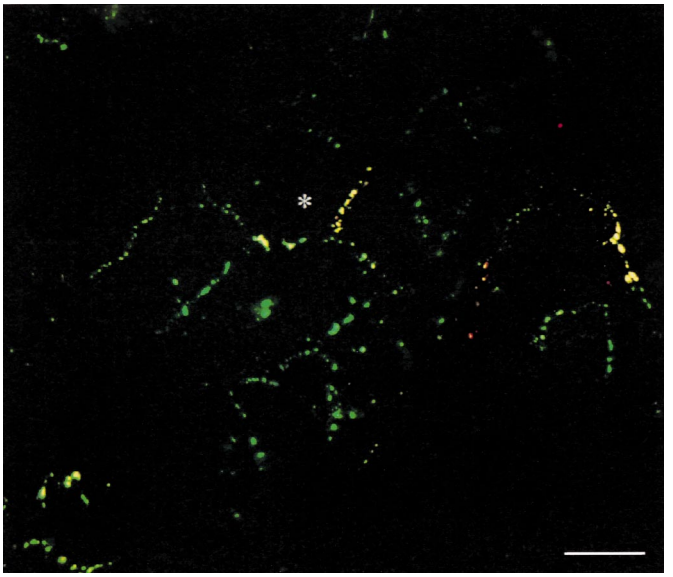

Fig. 3. Distribution of Cx43 and Cx40 in cultured neonatal rat ventricular myocytes. Fluorescent image of neonatal rat ventricular myocytes cultured at >75% confluency for 48 h double-labeled with mouse anti-Cx43 (green) and rabbit anti-Cx40 (red). As can be concluded from the frequent yellow and only sparse red labeling, most Cx40 is colocalized with Cx43. Bar represents 25 μm.

Most of the cells, including anti-Cx40 labeled cells, were positive for the myocardial marker desmin and their contractile phenotype was sometimes established by a clear cross-striation indicative for cardiac muscle cells. However, by isolating cells from whole ventricle some contamination of non-muscle cells like endothelial cells or fibroblasts may be expected, although the bulk of fibroblasts was removed from the cultures by including a differential plating step of 1 h in the isolation procedure [40]. In order to exclude the possibility that Cx40 labeling is confined to vascular endothelial cells, double labeling experiments were performed where mouse anti-desmin or anti-vWf antibodies were combined with rabbit anti-Cx40 antibody. Cultures of neonatal rat ventricle incubated with anti-vWf antibody revealed no positive staining (not shown), whereas immunolabeling could be observed in cultures of human umbilical cord endothelial cells using a similar procedure [42]. This may indicate that endothelial cells were removed during the differential plating step or died during the enzymatic isolation of cells. Double-labeling of mouse anti-desmin with rabbit anti-Cx40 antibodies revealed anti-Cx40 immunoreactivity in anti-desmin positive cells (e.g. Fig. 2C–F).

All immunostainings that were performed on myocyte cultures at >75% confluency showed a homogenous pattern of anti-Cx43 labeling and a heterogeneous pattern of anti-Cx40 labeling. The reduction of culture density from >75% confluency to ~50% confluency, leads to a reduced percentage of cell–cell contacts which were anti-Cx40 positive in the latter cultures (compare Fig. 2C,D with E,F). Cx43 immunostaining showed no gross alterations between both culture densities (not shown). Note that the muscle cell pair in the middle of Fig. 2E,F shows no anti-Cx40 staining. A further reduction to <25% confluency allowed us to observe many isolated cell pairs (see Fig. 5, left upper panel). We never observed Cx40 staining between the more than 50 desmin-positive cell pairs we examined (see Fig. 4A,B), whereas Cx43 is clearly detected between such cell pairs (see Fig. 4C,D). In double-labeling experiments performed with mouse anti-Cx43 and rabbit anti-Cx40 antibodies this point is even further stressed (Fig. 4E,F).

In an attempt to quantify these apparent differences in Cx40 expression in high and low density cultures we counted the relative number of Cx40 positive interfaces under both conditions and compared this with the relative number of Cx43 positive interfaces in parallel cultures (Table 1). Whereas the relative number of Cx43 positive interfaces was identical at both densities (95%), the relative number of Cx40 positive interfaces was considerably less in low density cultures (35%) compared to high density cultures (70%).

Western blotting was performed in order to compare the levels of Cx40 expression at different culture densities. Proteins were extracted from cultures of neonatal rat ventricular myocytes grown to <25, ~50 and >75% confluency (see Fig. 5 top panels). From cultures of each density equal amounts of total protein were blotted and probed with anti-Cx40 or anti-Cx43 antibody. Subsequent chemiluminescence detection revealed for Cx40 at all three densities a single band with a M_r of ~42 kDa (Fig. 5, αCx40, lanes 1–3). This protein could not be detected in control blots incubated with the primary antibody preincubated with the immunogenic peptide (Fig. 5, αCx40+ peptide, lanes 1–3). Although the band detected in lane 1 (representing cultures of <25% confluency) may seem

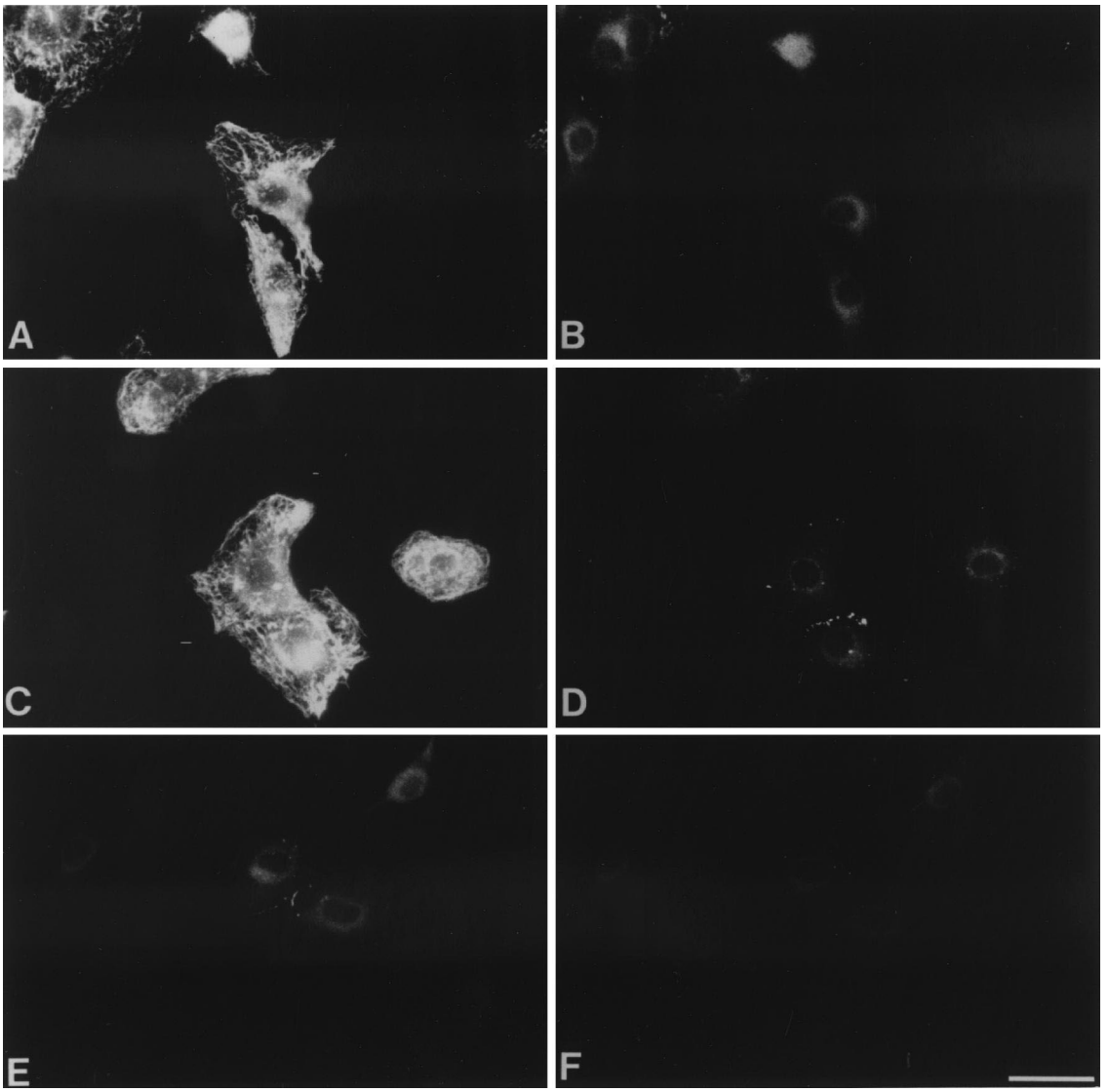

Fig. 4. Distribution of Cx43 and Cx40 in cultured neonatal rat ventricular myocyte pairs. Fluorescence images of neonatal rat ventricular myocytes cultured at <25% confluency for 48 h showing cell pairs double-labeled with mouse anti-desmin (A) and rabbit anti-Cx40 (B), or mouse anti-desmin (C) and rabbit anti-Cx43 (D), or mouse anti-Cx43 (E) and rabbit anti-Cx40 (F). Bar represents 25 μm.

slightly less intense, no clear differences in the intensity of the 42-kDa band in any of the three lanes were observed in repetitive experiments. An unspecific band of unknown identity at ~65 kDa was observed with and without peptide preincubation in the samples from cultures at ~50% confluency. For Cx43 a single band with a M_r of ~45 kDa and of equal intensity could be detected at all three densities (Fig. 5, αCx43, lanes 1–3).

Table 1
Gap junctional expression of Cx43 and Cx40 in neonatal rat ventricular myocytes cultured at various densities for 48 h[a]

Cx	Culture density	Number of positive interfaces	Total number of counted interfaces	Relative number of positive interfaces (%)
Cx43	High	398	420	95
Cx43	Low	96	101	95
Cx40	High	380	546	70
Cx40	Low	45	129	35

[a] Cx40 or Cx43 labeling at cell–cell interfaces in cultures of low (<25% confluency) and high (>75% confluency) density was counted and divided by the total number of cell–cell interfaces present in this particular area of the coverslip.

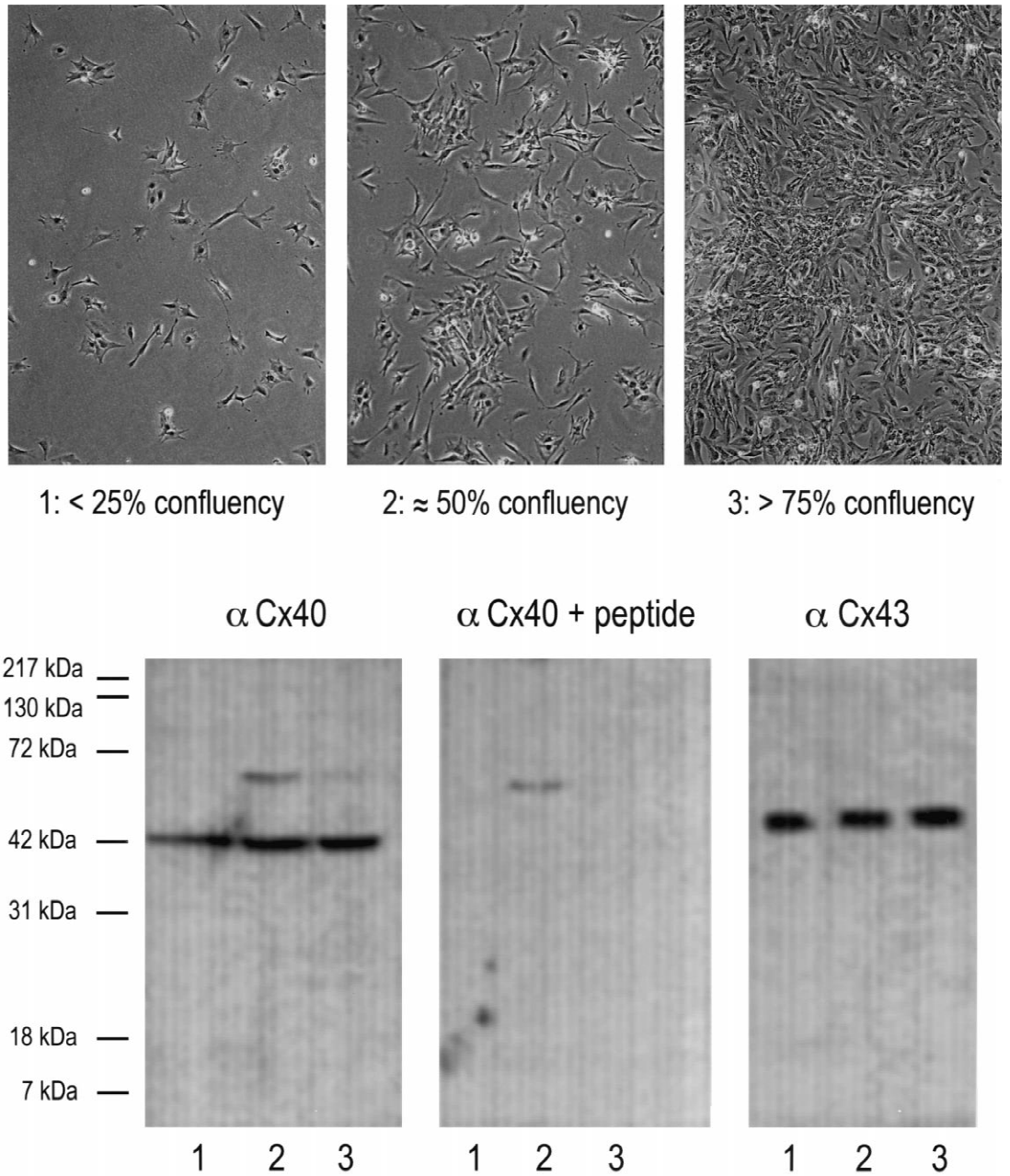

Fig. 5. Expression of Cx40 and Cx43 in neonatal rat ventricular myocytes cultured at various densities for 48 h. Top panels are representive phase-contrast photographs illustrating the different culture densities used. Lower panels show Western blots with Cx40 antibody (left), Cx40 antibody after preabsorption with its immunogenic peptide (middle) and Cx43 antibody (right). Lanes 1: <25% confluency, lanes 2: ~50% confluency and lanes 3 >75% confluency. Equal amounts of total protein (50 μg) were loaded per lane on 12.5% SDS-PAGE and the connexins were detected by immunoblotting. For Cx40, at all three densities a single band at ~42 kDa was detected. This band, which represents Cx40, was not detected in blots incubated with the Cx40 antibody preincubated with the immunogenic peptide. An unspecific band of unknown identity at ~65 kDa was observed with and without peptide preincubation in the samples from cultures at ~50% confluency. For Cx43, at all three densities a single band at ~45 kDa was detected. Migration of molecular mass markers is indicated on the left of the figure.

4. Discussion

The homogeneous expression of gap junction protein Cx43 in the ventricular working myocardium of neonatal rats is unambiguously shown, both in sections of whole hearts and in isolated cardiomyocytes [15,16,26]. However, recent studies on the expression of Cx40 in embryonic and fetal hearts revealed a dynamic and heterogeneous distribution pattern during development that has not yet fully matured in the neonatal heart just after birth [23,34]. Cx40 expression is most extensive in the fetal period. Towards birth Cx40 mRNA gradually disappears

from the rat ventricular myocardium; from the ventricular free wall towards the trabeculations. Just after birth Cx40 mRNA is still expressed in a subendocardial layer in the ventricles and in the atria. In the adult rat, Cx40 is only detectable in the conduction system and has disappeared from both the atrial and ventricular working myocardium [24]. The distribution pattern of Cx40 protein we report in this study confirms the data on the Cx40 mRNA distribution pattern. Moreover, it emphasizes the dynamic and heterogeneous nature of the ventricular myocyte population with respect to Cx40 expression. The age of the neonatal rats used to obtain ventricular cell cultures may thus determine the proportion of cells able to express Cx40 in those cultures. We have attempted to standardize for this variable in our experiments by using rats between 24–48 h after birth.

Oyamada et al. [38] studied dye coupling and Cx43 expression in confluent cultures of neonatal rat ventricular myocytes over a period of 7 days [38]. They observed an increase both in cell-to-cell coupling and in Cx43 expression with time, which occurred mainly between day 3 and day 7. We have performed immunocytochemical stainings on ventricular myocyte cultures of three different densities ranging from very low density (<25% confluency) to subconfluent (>75% confluency). In all cultures Cx43 and Cx40 could be detected. Moreover, for a given density identical Cx43 or Cx40 staining patterns were observed at 24, 48 and 96 h of culturing. Immunolabeling experiments were initially performed at >75% and ~50% confluency and showed a homogeneous pattern of anti-Cx43 labeling and a heterogeneous pattern of anti-Cx40 labeling. However, in electrophysiological recordings (dual whole-cell or perforated patch voltage-clamp) performed on cell pairs in cultures of <25% confluency we did not observe any indication of the presence of Cx40 gap junction channels between ventricular cell pairs (e.g. [43,44]). The observed single channel conductances were ~20, 40–45, and 70 pS which are indicative for Cx43 gap junction channels [8,45,46]. The expected size for Cx40 gap junction channels, on the other hand, would be 120–160 pS [47–49]. Events of this size were not observed in our recordings. In an attempt to explain this apparent controversy, we performed immunostaining experiments on ventricular myocyte cultures of low density (<25% confluency) as also used for dual patch clamp studies. The pattern of Cx43 immunoreactivity was not affected by the culture density. In contrast, in ventricular myocyte cultures of low density the relative number of cell–cell interfaces that were Cx40-immunopositive decreased as compared to high density cultures. Moreover, Cx40 staining was detected in these cultures only between clusters of three or more cells and not between cell pairs. A possible explanation for this phenomenon may be that isolated neonatal rat ventricular cells are all able to express Cx40, however, this expression is upregulated with increasing culture density. In other words, cells may only express Cx40 if they are in close contact with their neighboring cells. In an attempt to verify such density-dependent expression of Cx40, Western blots detecting Cx43 or Cx40 from equal amounts of total protein obtained from cultures grown at three different densities were carried out. Although the 42-kDa band in lane 1 of Fig. 5 (representing cultures of <25% confluency) may seem slightly less intense, no clear differences could be observed in the intensity of the 42-kDa bands in the three lanes in repetitive experiments. Indeed, cultures of <25% confluency contain many cell pairs, however, many small clusters of three to six cells are also present. These clusters express Cx40 (a part of such a cluster can be seen at the left top corner of Fig. 4A,B) and thus may account for the Cx40 protein detected in Western blots of these cultures. In addition, the possibility that some of the Cx40 detected in Western blots is from intracellular (non-plasmalemmal) origin cannot be excluded. An alternative explanation for the density-dependent expression of Cx40 may be that only a relative small proportion of subendocardial cells from the total ventricular myocyte population still have the ability to express both Cxs. Actual formation of Cx40 gap junction channels will then only occur in the case that two neighboring cells form Cx40 hemichannels. The probability that this occurs is much more frequent in clusters, where cells have several neighbors (sometimes up to eight), than in a cell pair or triplet. Observing anti-Cx40 immunolabeling in one myocyte exclusively at the cell–cell contact with some of its neighbors and not with all of its neighbors will favor this hypothesis. Indeed, as can be observed in Fig. 3 (asterisk) this is sometimes the case. The indicated cell shows only Cx40 immunolabeling at the gap junction it formed with two of its neighbors (at the right and bottom side) and not at its gap junctions with three to four other neighboring cells, where, however, Cx43 immunolabeling is apparent.

In summary, it may be concluded that our cultured neonatal rat ventricular myocytes compare very well with cells from the intact ventricular myocardium with regard to their composition of gap junctions. They will thus be a good model for studying short-term and long-term regulation of cardiac gap junction channel expression and function. Care should be taken, however, in comparing experiments performed on high and low density cultures. Furthermore, the heterogeneity of Cx40 expression may account for large scatter in results of experiments performed on (sub)confluent cultures.

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