Cell-to-cell interaction prevents cell death in cultured neonatal rat ventricular myocytes

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Abstract

Objectives: Loss of cardiac cells and the anatomical or functional remodeling of intercellular coupling occur under several pathological conditions. We have assessed the significance of intercellular coupling for cell death. Methods and Results: Ventricular cells obtained from 1 day old Wistar rats were cultured. Apoptosis was detected by nick-end labeling. Cells were plated at low and high cell density (3×10⁶/ml and 12×10⁶/ml, respectively). Cultured myocytes died spontaneously by apoptosis in a time dependent manner. The increase of the apoptotic cell population in a culture with high cell density on day 4 (1±1.2%, n=4) was significantly lower than that in a culture with low cell density (20±5.5%, n=4). The progression of apoptosis in the culture of low cell density was prevented in part after application of the medium extract from the culture of high cell density; the apoptotic cell population on day 6 decreased from 57±8.0% (n=4) to 36±3.8% (n=4). Treatment of the cultured myocytes at high cell density with antisense oligonucleotide for connexin43 (Cx43) for 24 h on day 2 resulted in a significant decrease in Cx43 expression as judged by Western blot, dye transfer and immunocytochemistry using mouse monoclonal antibody for Cx43. In association with the down-regulation of Cx43, the progress of apoptosis was accelerated; the apoptotic cell population on day 5 in the antisense-treated cultures (27±7.8%, n=4) was significantly higher than the sense-treated cultures (5±1.1%, n=4). The effect of Cx43 antisense treatment to promote apoptosis was not reversed by application of high cell-density culture medium. Conclusions: These findings suggest that cell–cell communication through gap junction formation and some humoral factors play important roles in the survival of cultured myocytes.

Keywords: Apoptosis; Cell communication; Gap junctions; Myocytes; Cell culture/isolation

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1. Introduction

Apoptosis is observed during myocardial infarction, heart failure, and ischemia-reperfusion [1–4]. Under such pathological conditions, a variety of functional and morphological abnormalities of gap junction organization has also been recognized [5–8].

Adult cardiac myocytes are fully differentiated and non-proliferating cells, and survive throughout the life of the animal. A variety of signals required for cell survival have been reported by previous investigators [9,10]. Survival of nerve cells depends on several neurotrophic factors including nerve growth factor, and the depletion of these factors results in apoptotic death of neurons [11]. In cardiac cells, some growth factors such as cardiotrophin-1 [12,13] or insulin-like growth factor [14] were shown to inhibit apoptosis. In addition to these humoral factors, cell–cell...
adhesion proteins or the interaction with extracellular matrix have also been suggested to play an important role in regulating cell survival [15–20]. Cardiac cells in the heart in-vivo are connected with each other electrically and metabolically by gap junctions, and gap junction formation is considered to be essential for propagation of excitation [21], as well as for normal development and growth of the heart [22]. Unlike cardiac cells in the heart in-vivo, isolated cardiac myocytes in culture cannot survive for a long period, and they die spontaneously with time by apoptosis [23]. Among a variety of culture conditions for cell survival, gap junction formation could be an important one.

The present study was designed to shed light on cell-to-cell interaction in prevention of cardiac apoptosis. We examined the effects of cell density, humoral factors and gap junction formation on the time-dependent progression of apoptosis in cultured neonatal rat ventricular myocytes.

2. Methods

2.1. Cell isolation and culture

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).

Single ventricular myocytes were obtained from 1 day neonatal Wistar rats by methods described previously [24]. Briefly, the ventricles were dissected from ten neonatal rats and cut into pieces. The ventricular pieces were incubated in the Dulbecco’s phosphate-buffered saline containing 0.16 mg/ml collagenase (Yakult, Japan) with stirring at 37°C for 15 min, and the cell suspension was collected. This procedure was repeated four times. The pooled suspension was centrifuged at 1000 rpm for 5 min, and cells isolated were cultured in M199 (GIBCO, USA) with 10% fetal bovine serum (GIBCO), 5 μM cytosine arabinoside, 50 μg/ml penicillin and 50 μg/ml streptomycin at 37°C in a humidified CO2 incubator. We plated isolated ventricular cells at low and high cell densities (3 × 10^4/ml and 12 × 10^4/ml, respectively). Cultured myocytes were fixed in 4% paraformaldehyde for 10 min in phosphate buffered saline for cell staining.

2.2. Detection of apoptosis

DNA fragmentation in cultured cells was assessed in situ by terminal deoxynucleotidyl transferase (TdT)-mediated digoxigenin-deoxyuridine triphosphate (dUTP) nick-end labeling using FITC-based, direct immunocytochemistry. Nick-end labeling (TUNEL) was performed using Apoptag kit (Oncor Inc., Gaithersburg, MD, USA). In this study we defined a TUNEL positive cell as cell death through apoptosis, although this method is controversial for detection of apoptosis [25]. Fluorescence was evaluated using a Zeiss microscope Axiohot 2 (Carl Zeiss, Jena, Germany). Approximately 300 cardiocytes attached on each cover glass were observed by random sampling, and the numbers of cells stained and unstained with nick-end labeling were counted. The data from different days in culture were obtained from four experiments and averaged.

To confirm the reliability of the TUNEL method in apoptosis detection, we performed nuclear staining by Hoechst 33342 (Molecular Probes, OR, USA) in the experiments to see the effect of cell density on apoptosis. Cultured cells were stained with 20 μM Hoechst 33342 for 10 min. Numbers of cells with and without chromatin condensation, a characteristic change of apoptosis [26], were counted.

2.3. Immunocytochemistry for Cx43 or α-sarcomeric actinin

Nick end labeling and Hoechst 33342 staining were followed by the staining for Cx43 or α-sarcomeric actinin. Cultured cells were incubated with mouse anti-Cx43 monoclonal antibody (Chemicon, Temecula, CA, USA) overnight at 4°C (diluted 1:200 in PBS) or mouse anti-α-sarcomeric actinin antibody (Sigma, St.Louis, MA, USA) for 30 min (diluted 1:800 in PBS) at room temperature. They were then incubated with goat anti-mouse IgG labeled Texas Red (Southern Biotechnology Associates, Inc., Birmingham, AL) for 60 min (diluted 1:200 in PBS). The α-sarcomeric actinin staining was used to identify cardiac myocytes. Most of the cultured cells (~ 95%) were identified as cardiac cells. In control experiments without the primary antibodies, significant fluorescence was not detected.

2.4. Treatment of cells with oligonucleotides

The Cx43 antisense oligonucleotide (5’-CTCCAG-TACCCTGCTG-’3’) was purchased from Life Technologies (Tokyo, Japan). The sequence is complementary to the AUG translation start codon (underlined) and upstream and downstream nucleotides of murine Cx43 mRNA [27]. As a control, the corresponding sense oligonucleotide (5’-CAGACATGGTGACTGGG-’3’) was also purchased from Life Technologies. The oligonucleotides were dissolved in sterile water and stored at ~80°C. The oligonucleotides were mixed with the liposomes (Lipofectin, GIBCO/BRL, Gaithersburg, MD, USA) and incubated for 30 min at room temperature, then added to serum-free medium. The final concentrations of oligonucleotides and liposomes were 2.5 μM and 4 μg/ml, respectively. Cultured myocytes with high cell density on day 2 were washed with serum-free medium and then re-fed with the liposome-oligonucleotides medium. The cells were incubated at 37°C for 24 h and then washed and re-fed with serum-containing medium. Fixation of the cells
for immunocytochemistry or extraction of proteins for Western blotting was then carried out.

2.5. Western blotting

Cell lysates (50 μg of total protein measured using BCA protein assay) were loaded on 10% SDS polyacrylamide gels. Proteins on gels were electrotransferred to PVDF membrane. Membranes were immersed in blocking buffer (5% low fatty milk, 0.1% Tween20, 0.02% NaN₃ in PBS) for 60 min and then subjected to primary antibodies against cadherin and actin (rabbit anti-actin polyclonal antibody, Sigma, St. Louis, MO, USA) for 90 min (diluted 1:1000 and 1:1000 in PBS, respectively) at room temperature. Primary antibodies were detected by incubation with horseradish peroxidase-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) for 60 min (diluted 1:10000 in PBS) at room temperature, followed by enhanced chemiluminescence (ECL) (Amersham, Buckinghamshire, UK). For connexin immunoblotting, membranes were stripped of bound antibodies. Membranes were incubated in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7) at 50°C for 30 min and then washed in PBS containing 0.1% Tween20 twice for 10 min. Membranes were reprobed by incubation with primary antibody for Cx43, connexin45 (Cx45) or connexin40 (Cx40), overnight at 4°C. The mouse anti-Cx43 monoclonal antibody (Chemicon, Temecula, CA, USA) was used at 1:1000 dilution. The guinea pig anti-Cx45 antibody (GP42) and the mouse anti-Cx40 antibody (R83) were generous gifts of Dr. Nicholas J. Severs. GP42 and R83 were used at 1:50 dilution and at 1:100 dilution, respectively. The primary antibodies for Cx43 were detected by horseradish peroxidase-labeled goat anti-mouse IgG (Transduction Laboratories, Lexington, KY, USA) (incubation for 60 min at room temperature, diluted 1:2000 in PBS) and ECL. Detection of GP42 and R83 were performed with horseradish peroxidase-labeled anti-guinea pig IgG (Chemicon, Temecula, CA, USA) and horseradish peroxidase-labeled goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) (incubation for 60 min at room temperature, diluted 1:1500 in PBS), respectively. Films of Western blotting were digitized with an Epson GT-9500 image scanner (Epson, Japan). The density of each protein band was quantified by NIH image software and then calculated as the ratio of connexin or cadherin to actin. The values for each sample are presented in percentage of control (sense treatment) on the same gel. Mean values were derived from 4 to 6 different experiments.

2.6. Intercellular dye-transfer

Gap junctional intercellular communication was assessed by a microinjection dye transfer method [28]. Cultured medium was replaced with PBS containing 0.1 mM CaCl₂. Glass capillaries (Sterile femtotips 5242, Eppendorf) were filled with 10% solution (w/v) of Lucifer Yellow dissolved in 0.33 mM LiCl₂ (pH = 7.2, 10 mM HEPES). Lucifer Yellow was injected into cultured myocytes by a microinjector (Transinjection 5246, Eppendorf). Two minutes after the microinjection, cultured cells loaded with dye were counted by epifluorescence and phase-contrast view. Experiments were carried out at room temperature.

2.7. Data analysis

Experimental data are expressed as mean±SEM. Statistical analysis was performed using Student’s t-test with P<0.05 as the level of significance.

3. Results

3.1. Effects of cell density

We first investigated the effect of cell density on occurrence of cell death (apoptosis) in cultured myocytes. The cells cultured with low cell density were sparse and cell–cell contact was infrequent (Fig. 1A top and Fig. 2A, top and middle). The cells cultured with high density were in a subconfluent state (Fig. 1A, bottom and Fig. 2A, bottom). In the low density cultures, both beating and non-beating cells coexisted (approximately half of the myocytes were spontaneously beating). In the high density cultures, most cells contracted spontaneously and regularly, but their beating rate varied at different locations. In the low density cultures, positive cells for nick-end labeling (apoptotic cells) were more prevalent than in the high density cultures. The results obtained in four experiments are summarized in Fig. 1B. In the low density cultures, the population of positive cells for nick-end labeling increased progressively during cultivation for 6 days. In contrast, there was no substantial increase in the population of apoptotic cells in the high density cultures. The population of the apoptotic cells on day 4 and day 6 in cultures with low density were 20±5.5% (n=4) and 57±8.0% (n=4), respectively, whereas it was virtually zero during cultivation with high density.

It was also found in experiments with Hoechst 33342 staining that the population of apoptotic cells on day 4 showing chromatin condensation in low density cultures (31±2.1%, n=4) was significantly higher than that in high density cultures (4.5±0.4%, n=4) (Fig. 2).

3.2. Effects of humoral factors

To examine the effects of humoral factors released from cultured cells, we replaced the medium of the low density cultures by the medium of high density cultures (cultivation for 2 days). This medium exchange was performed on day 2 and on day 4, and the effects were monitored after
Fig. 1. Nick-end labeling in cultured myocytes at different cell densities. (A) Cardiac apoptosis was observed in the low cell density culture (day 4), but not in the high cell density culture (day 4). Green fluorescence indicates nuclear DNA fragmentation stained with the nick-end labeling (a marker of apoptosis). α-sarcomeric actinin is labeled in red. Calibration bar=50 μm. (B) Changes in population of apoptotic cells during cultivation for 6 days. Open symbols indicate low density cultures. Closed circles indicate high density cultures. Open triangles indicate low density cultures supplemented with the conditioned medium of high density cultures. Values (percentage of cell population) are means±SEM of each four experiments. *P<0.05 vs. high cell density cultures (●). **P<0.05 vs. low cell density control cultures (○).

3.3. Effects of gap junction formation

Cell–cell communication through gap junctions could also be involved in the prevention of apoptosis in the high density cultures. Therefore, cells cultured with high cell density were exposed to Cx43 antisense oligonucleotide on day 2 for 24 h. Western blotting on day 5 showed three bands of Cx43 proteins either in Cx43 antisense-treated cells or in Cx43 sense-treated cells, indicating two phosphorylated (P1, P2) forms and a nonphosphorylated (NP) form of the protein (Fig. 3). The relative amounts of P1, P2 and NP were 36±3.9%, 28±5.9% and 35±7.5% (n=6), respectively in the Cx43 sense-treated cells. The total density of these three Cx43 protein bands was normalized to that of actin. Compared with the sense-treated cultures (100%), the amount of Cx43 in the antisense group was decreased to 67±13.9% (P<0.05, n=6). The relative amounts of P1, P2 and NP were not effected by the antisense treatment (37±2.9%, 24±5.1%, 39±7.9%, respectively, n=6).

The influence of Cx43 antisense treatment on the expression of Cx45 and Cx40 was examined by Western blotting. Compared with sense-treated cultures (100%), the amount of Cx45 in the antisense-treated cultures was unchanged (97±0.4%, n=4). Cx40 was neither detected in the sense group, nor in the antisense group.

Fig. 4 shows expression of Cx43 in cultured myocytes on day 5 using immunocytochemistry. The myocytes were also stained by nick-end labeling. In Cx43 sense-treated cultures, Cx43 protein was expressed as a continuous line or punctuated spots (red) in the border of two adjacent cells. In Cx43 antisense-treated cultures the expression of Cx43 was minimal. On the other hand, the population of...
cells positive for nick-end labeling (green) was minimal in the sense-treated cultures (5±1.1%, n=4), but more abundant in the antisense-treated cultures (27±5.7%, n=4) (P<0.05). All the positive nick-end labeling was recognized in the cells away from the positive Cx43 labeling. These findings suggest that decreased gap junction density was associated with an increase in cell death.

In Western blotting (Fig. 3B), there was no difference in cadherin expression between the Cx43 antisense and sense groups. This means that cadherin-mediated cell–cell contact was unaffected by Cx43 antisense.

Fig. 5 shows the effect of Cx43 antisense oligonucleotide on gap junctional communication assessed by dye-transfer. Lucifer Yellow was introduced into individual cultured myocytes on day 5 by direct cell injection. Cells, comprising Lucifer Yellow transported by intercellular communication via gap junctions, were quantified. The number of the dye-loaded cells in the antisense group (8±2.2, n=8) was significantly smaller than that in the sense group (18±1.3, n=9), indicating reduction of gap junctional communication induced by Cx43 antisense treatment.

The enhancement of apoptosis by Cx43 antisense treatment might be the result of less production of apoptosis-inhibitory humoral factors. This possibility was tested by application of the medium from normal (untreated) high density cultures for 48 h to Cx43 antisense-treated cultures on day 3. The population of apoptotic cells (positive nick-end labeling) in the antisense-treated cultures with the medium application (28±3.5%, n=4) was not significantly different from that in the cultures without the medium application (33±6.6%, n=4). This observation suggests...
Fig. 3. (A) Representative Western blots of Cx43 gap junction protein and cadherin in Cx43 antisense oligonucleotide (AS) and sense-treated cells (S). The cells were incubated with Cx43 antisense or sense oligonucleotides (2.5 μM) in liposomes for 24 h on day 2 and sampled on day 5. The nonphosphorylated (NP) and phosphorylated (P1 and P2) forms of Cx43 are marked. Cell lysates (50 μg) were loaded on gels. (B) Mean data of Cx43 and cadherin content in six Cx43 sense-treated and six Cx43 antisense-treated cell cultures. The ratio of Cx43 or cadherin band density to actin band density was calculated and then normalized by the value of sense-treatment. Data are presented as mean±SEM. *P<0.05 AS vs. S.

Fig. 4. Effects of Cx43 antisense treatment on the Cx43 expression and apoptosis. The cells were treated Cx43 antisense (AS) or sense oligonucleotide (S). Cells were fixed on day 5. (A) Double staining for nuclear DNA fragmentation (green) and Cx43 gap junction protein (red) in upper panels. In the lower panels, cell margins are traced and nuclei are pasted in gray color. Arrows indicate DNA fragmentation. Calibration bar=50 μm. (B) Percentage of myocytes stained with nick-end labeling. Data were obtained from four different experiments, and are expressed as mean±SEM. *P<0.05 AS vs. S.
Fig. 5. Effects of Cx43 antisense on gap junctional intercellular communication of cultured myocytes. The Cx43 antisense or sense oligonucleotides were applied to cells on day 2 for 24 h. On day 5, individual cells were injected with Lucifer Yellow. The number of the dye-loaded cells was measured 2 min after microinjection. Left and right panels show the sense-treated and the antisense-treated cells, respectively. Arrowheads indicate the injected cells. Calibration bar=100 μm.

that the influence of apoptosis-inhibitory humoral factor on the antisense-treated high density culture might be minimal or negligible (see Discussion).

4. Discussion

Key observations in the present study are as follows: (1) Myocytes cultured at low cell density are prone to premature cell death, compared with cells at high cell density; (2) The conditioned medium of the high density cultures prevented this phenomenon in part; (3) Inhibition of gap junction formation through Cx43 antisense treatment increased cell death. These findings suggest that one or more humoral factors secreted from cardiac myocytes as well as gap junction competence are important for survival of cells in culture.

4.1. Gap junctions

Apoptosis is a type of cell death through a specific cell suicide program [29]. This machinery is conserved in mammalian cells, and activated by various physical and chemical stresses. A proteolytic system involving caspases is considered to be a key factor in signal transduction for apoptosis. Gap junction channels play an important role in the cell to cell transfer of ions and small molecules (~1 kDa) such as cyclic AMP, Ca\(^{2+}\), and inositol triphosphate [21]. These molecules are reported to trigger the cell suicide program [30–32]. In this study, we demonstrated that Cx43 antisense oligonucleotide not only suppressed the expression of Cx43 protein and the functional gap junctional communication, but also increased cell death. In cardiac cells coupled with neighbors, an elevation of apoptotic triggers induced by various stimuli in a certain cell (or group of cells) may be diluted by cytosolic communication with healthy neighbors through gap junctions. Such a protecting mechanism may be inhibited by the antisense treatment for Cx43.

Cx43 is the principal connexin of gap junction proteins in the mammalian heart [27]. Abundant expression of Cx43 protein was observed in high density cultures (Fig. 2, 4). Darrow et al. [33] reported that connexin45 (Cx45) protein is expressed in cultured rat ventricular myocytes in addition to Cx43 protein. Gap junctions composed of Cx45 could also be involved in control of cultured cell survival. In the present study Cx43 antisense treatment of high density cultures did not affect the Cx45 protein expression. Thus, we cannot rule out possible contributions of Cx45 gap junctions to the survival of the cells.

4.2. Humoral factors

The high cell-density culture medium may contain some humoral factors which prevent apoptosis. Cardiotrophin-1 (CT-1) and fibroblast growth factor (FGF) could be candidates. It was shown by Sheng et al. [13] that CT-1 application to neonatal cultured ventricular cells promotes their survival. Cuevas et al. [34] reported that application of FGF prevents cardiac cell apoptosis induced by ischemia-reperfusion. In the present experiments, cell density of the culture may have affected production of these apoptosis-inhibitory humoral autocrine or paracrine factors. However, it should be noted that other types of humoral factors exist.

The medium of untreated high density cultured myocytes failed to prevent apoptosis in the Cx43 antisense-treated high density myocyte culture. Possible explanations for these results include but are not restricted to the following: (1) apoptosis within cultures showing reduced...
gap junctional communication cannot be rescued by humoral factors, or high density culture may be insensitive to the humoral factors, (2) humoral factors are not involved in the apoptosis we observed i.e. cell to cell communication rescues cells from apoptosis. This correlates with our observation that higher numbers of non-contacting myocytes experienced apoptosis than those cells contacting their neighbors in both high density cultures which have been treated with Cx43 antisense oligonucleotides and those left untreated.

Apoptosis of cardiac myocytes is enhanced by angiotensin II [35,36], atrial natriuretic peptides [37], and tumor necrosis factorα [38]. As to the role of these apoptosis-promoting humoral factors in the cell density-dependent cell survival, no information is available at the moment. Further experimental studies will be required to specify the apoptosis-affecting humoral factors and to clarify their mode of actions.

4.3. Limitation

In the present experiments, most of myocytes were spontaneously beating in the high density cultures, whereas approximately half of the myocytes were spontaneously beating in the low density cultures. Accordingly, differences in the extent of functional and morphological cell differentiation might also have affected the cell survival through unknown mechanisms. With respect to this point, Clark et al. reported that in cultures of adult myocyte cell–cell contact promotes the development of spontaneous activity and cell differentiation, and extends myocyte survival [39].

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