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Connexins and apoptotic transformation

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Abstract

We examined the influence of connexin (Cx) expression on the development of apoptosis in HeLa parental cells (coupling deficient cell line) and HeLa cells expressing wild-type Cx43 and Cxs fused with enhanced green fluorescent protein (EGFP). EGFP was attached to the C-terminus of Cx32 and Cx43, Cx32-EGFP and Cx43-EGFP, respectively, and to the N-terminus of Cx32, EGFP-Cx32. All fusion proteins assembled into junctional plaques (JPs) at areas of cell–cell contact, but only the C-terminal fusion proteins formed functional gap junction (GJ) channels as well as hemichannels. In each cell line, apoptosis was induced by treatment with various agents including anisomycin, camptothecin, *cis*-platinum, colchicine, cycloheximide, etoposide, staurosporin and taxol. Using fluorescence microscopy, time-lapse imaging and dual whole-cell voltage clamp techniques, we correlated the changes in functional properties of GJ channels and Cx distribution with the progression of apoptosis based on cells' labeling with acridine orange and ethidium bromide (EB). The early phase of apoptosis (a viable apoptotic (VA) state) was characterized by shrinkage of the cells and by increased internalization of JPs accompanied by decreased cell–cell coupling. The apoptotic reagents had no direct effect on electrical cell–cell coupling. Transformation from a VA to a nonviable apoptotic (NVA) state was faster in HeLa cells expressing Cx43 or Cx43-EGFP than in HeLa parental cells. The potent GJ uncoupler, octanol, slowed the transition of HelaCx43-EGFP cells into a NVA state. In the absence of apoptotic reagents, the rate of EB uptake was higher in HeLaCx43-EGFP than in HeLa parental cells consistent with the presence of open Cx43-EGFP hemichannels. However, in both cell lines the rate of EB uptake decreased proportionally during the development of apoptosis suggesting that membrane permeability ascribed to Cx hemichannels is reduced. Cells expressing Cx32-EGFP and EGFP-Cx32 demonstrate the same apoptotic patterns as HeLaCx43-EGFP and HeLa parental cells, respectively. Intracellular levels of ATP in HeLaCx43-EGFP cells were substantially lower than in HeLa parental cells, and ATP added to the medium abolished the accelerated transition from a VA to a NVA state in HeLaCx43-EGFP cells. In summary, Cx32 or Cx43 accelerates transformation of cells into a NVA state or secondary necrosis and this depends on the ability of Cxs to form functional GJ channels and hemichannels.

Keywords: Connexin; Apoptosis; Green fluorescent protein; Secondary necrosis; Intercellular communication

1. Introduction

Apoptosis is a genetically controlled process of programmed cell death that occurs during various stages of development and serves many vital functions, such as the control of cell number in continuously renewing cell populations, selective immune cell deletion, etc. [1]. Apoptosis is a complex physiological process that involves different intracellular signal transduction pathways including protein kinase cascades, pro-caspases, executioner caspases and a number of small molecules such as reactive oxygen species [2,3], Ca^{2+} and cAMP [4–6]. Most studies of apoptosis have focused predominantly on intracellular signaling pathways, but tissue function often involves intercellular (cell–cell) communication, which comes in several forms. We focused on the form of cell–cell communication mediated by GJ channels, which provide a direct pathway for electrical and metabolic signaling between neighboring cells [7–10]. GJ channels are composed of protein subunits, termed Cxs, a large family of

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Abbreviations: EGFP, enhanced green fluorescent protein; Cx, connexin; GJ, gap junction; JP, junctional plaque; V, viable non-apoptotic cells; VA, viable apoptotic cells; NEC, necrotic cells; NVA, nonviable apoptotic cells; ChF, chromatin-free cells; AO, acridine orange; EB, ethidium bromide.

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homologous membrane proteins that includes 20 distinct isoforms in humans [11]. Each GJ channel is composed of two hemichannels (connexons), which in turn are composed of six Cx subunits. Oligomerization of connexins into hemichannels starts in the endoplasmic reticulum and is completed in the Golgi complex, where vesicles containing hemichannels travel or are transported to fuse with the plasma membrane [12]. The functional significance of GJ channels, as a pathway for intercellular signaling in a variety of tissues, is emerging from studies of targeted gene disruptions and of genetic diseases in humans [13–16]. There are well-known effects of gap junctional communication on cell proliferation and differentiation [17,18]. Mutations in connexins have been shown to be responsible for several hereditary human diseases including the X-linked form of CMT demyelinating diseases [19,20], non-syndromic sensorineural deafness [21], erythrokeratodermia [22], congenital cataractogenesis [23,24], cardiac malformation and defects of laterality [25]. A number of studies have also demonstrated a correlation between neoplastic transformation and reduced GJ communication. Recent studies proposed that intercellular communication through GJ channels might influence the development of apoptosis [26]. It has been suggested that triggering signals might be transferred through GJs thereby widening and, perhaps accelerating the spread of apoptosis [27,28]. Conversely, intercellular communication might rescue cells affected by pathological factors through interactions with healthy neighbors [29]. Diseases such as cancer, Alzheimers, atherosclerosis and ischemia are characterized by dysfunction of both, intercellular communication as well as apoptosis [27,30–32].

We examined apoptosis induced by a variety of chemical agents in HeLa cell lines stably expressing Cxs, either wild-type or fused with enhanced green fluorescent protein (Cx-EGFP) to determine whether connexin expression affected its induction and development. We show that HeLaCx43-EGFP cells exhibit a similar rate of transformation to a VA state compared to HeLa parental cells. However, HeLaCx43-EGFP cells appear to exhibit a faster transition from a VA state into a NVA state or a secondary necrosis. None of the agents used to induce apoptosis affected GJ channel conductance, but all reduced intercellular communication by blocking *de novo* GJ channel formation and speeding internalization of GJ channels from the junctional membrane. Our studies suggest that the accelerated transformation into a NVA state requires functional GJ channels and/or hemichannels.

2. Methods

2.1. Molecular cloning and cells transfection

Experiments were performed on HeLa parental cells, a human cervical carcinoma cell line (ATCC No. CCL-2) that is coupling deficient, and stable HeLa cell lines transfected with wild-type Cxs and Cx-EGFP fusion proteins. Protocols for transfecting HeLa cells with vectors encoding wild-type Cx43, as well as Cx32 and Cx43 fused with EGFP are as previously described [33].

2.2. Cell culture and apoptotic treatment

Cells were maintained in DMEM containing 10% FCS. Cells were grown to near confluence prior to treatment with agents that induce apoptosis. To assess how connexin expression affected induction of apoptosis, we treated cells with a number of apoptotic agents including: (1) anisomycin (1 μg/mL [34]), an inhibitor of protein synthesis at the translation step and activator of stress-activated protein kinases (SAPKs); (2) cycloheximide (10 μg/mL [35]), an inhibitor of protein synthesis; (3) colchicine (1 μg/mL [36]), a disrupter of microtubules and inhibitor of tubulin polimerization; (4) taxol (1 μM [36]), an antitumor and antileukemic agent that promotes microtubule assembly and inhibits tubulin disassembly; (5) camptothecin (CAM, 500 nM [37]), topoisomerase I inhibitor, an antileukemic and antitumor agent; (6) etoposide (50 μM [38]), topoisomerase II inhibitor, an antileukemic and antitumor agent; (7) *cis*-platinum (10 μg/mL [39]), a DNA crosslinking agent and antineoplastic drug; and (8) staurosporine (1 μM [40]), a potent broad-spectrum inhibitor of protein kinases. All these reagents were purchased from Sigma–Aldrich and prepared as stock solutions: 10 mg/mL of anisomycin in ethanol/DMSO (9/1), 10 mg/mL of cycloheximide in PBS, 1 mg/mL of colchicine in dH_2O, 10 mM of taxol in DMSO, 1 mM of CAM in DMSO, 50 mM of etoposide in DMSO, 1 mg/mL of *cis*-platinum in physiological solution, 1 mM of staurosporine in DMSO.

2.3. Apoptosis assay

Fluorescence microscopy (Olympus IX-70 microscope equipped with accessories for phase contrast and broad field fluorescence) was used to evaluate apoptosis. An apoptotic index was determined by nuclear condensation and segmentation, and by plasma membrane integrity using the two fluorescent dyes. AO was used to characterize chromatin condensation, and EB (Molecular Probes), to characterize membrane integrity. AO is membrane permeable and marks nuclei green, and EB, which binds to DNA, is mainly taken up by cells when membrane integrity is lost and stains nuclei red. Cells were grown as confluent monolayers, but trypsinized and centrifuged before evaluation. Cells were counted by using a hemacytometer and categorized into five groups based on AO and EB fluorescence in each individual cell; counting procedures are described in more detail in [41]. Cells were categorized as follows: (1) viable with non-apoptotic nuclei (V; bright green chromatin with organized

structure); (2) viable with apoptotic nuclei (VA; bright green chromatin that is highly condensed or fragmented); (3) necrotic (NEC; bright orange chromatin with organized structure); (4) nonviable apoptotic (NVA; bright orange chromatin that is highly condensed or fragmented); (5) chromatin-free (ChF; cells that totally lost their DNA content and are exhibiting a weak green–orange staining) cells.

2.4. Fluorescence imaging

Phase contrast and fluorescence microscopy with time-lapse imaging was used to record morphological changes, cell mobility and dye uptake over a period of ~48 hr. Fluorescence was monitored by using an imaging system equipped with an OlymPix-2000 cooled digital camera (12-bit), monochromator for fluorophore excitation, and UltraVIEW software (Perkin-Elmer) for image acquisition and analysis. Long-term time-lapse imaging (up to 4–5 days) was performed with cells plated in 35 mm Petri dishes with thin glass bottoms (MatTek Corporation). The Petri dishes were positioned into the micro-incubator adapted onto the stage of an inverted microscope, Olympus IX-70. The micro-incubator (constructed by Dr. F. Bukauskas) allows control of the composition and temperature of the atmosphere surrounding the Petri dish (normally it contains 5% CO_2), the temperature of the medium as well as exchange of the medium during imaging. Time-lapse imaging shows that cells grow normally for several days with a similar proliferation rate as that found in a standard CO_2 incubator.

2.5. Electrophysiological measurements

Cells on coverslips were transferred to an experimental chamber mounted on the stage of an inverted Olympus IX-70 microscope equipped with the fluorescence imaging system. The chamber was perfused with a modified Krebs–Ringer solution containing (in mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, 5 glucose, 2 pyruvate (pH 7.4). The dual whole-cell patch clamp was used to study the electrical cell–cell coupling (for details see [42]). Patch pipettes were filled with a solution containing (in mM): 10 NaCl, 140 KCl, 0.2 $CaCl_2$, 1 $MgCl_2$, 3 MgATP, 5 HEPES (pH 7.2), EGTA 2 ($[Ca^{2+}]_i = 5 \times 10^{-8}$ M). For data acquisition and analysis we used a MIO-16X A/D converter (National Instruments) and our own software.

2.6. ATP measurements

Intracellular ATP level of HeLa parental and HeLaCx43-EGFP cells was determined by the luciferin–luciferase method using a microplate reader Ascent FL (Labsystems). Statistical analysis was performed by calculating mean value and confidence interval (95%) and using SigmaPlot-2000 software.

3. Results

3.1. The time course of the development of apoptosis

To initially assess the effect of Cx expression on the development of apoptosis, we compared HeLa parental cells and HelaCx43-EGFP cells treated with anisomycin, 1 μg/mL. Typically HeLa cells expressing Cx43-EGFP exhibit punctate fluorescence in the cytoplasm as well as punctate and diffusional fluorescence in the plasma membrane (Fig. 1A). Within the junctional membrane (appositional area between two cells) regions of high

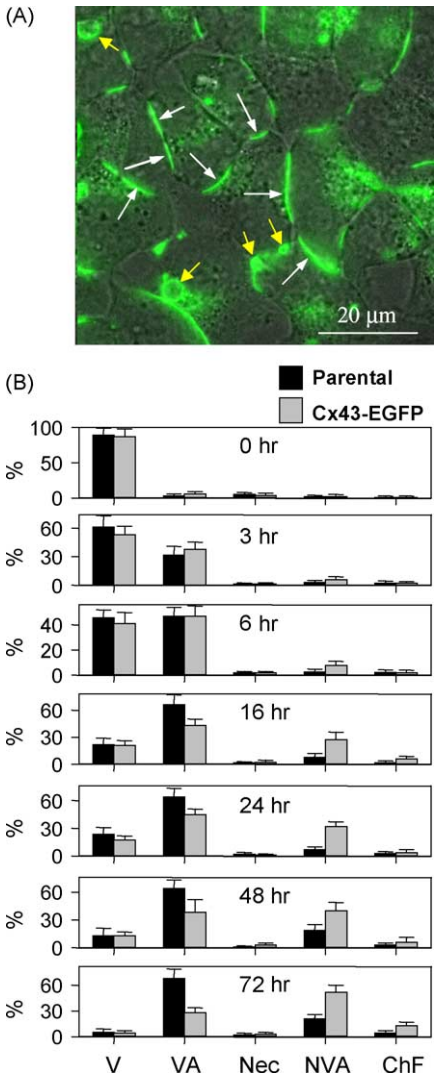

Fig. 1. Time course of the development of apoptosis measured in HeLa parental cells and HeLa cells stably expressing Cx43-EFGP. (A) Shown is an image of HeLaCx43-EGFP cells obtained by overlap of fluorescence (in green) and phase contrast (in gray) images. Cx43-EGFP forms large junctional plaques (white arrows) located at the appositional areas between neighboring cells. Yellow arrows indicate vesicles composed of internalized junctional plaques. (B) Shown are percentages of HeLa parental (black bars) and HeLaCx43-EGFP (gray bars) cells distributed among five categories (see Section 2). Apoptosis was induced by bath application of anisomycin, 1 μg/mL. The percentage of NVA cells was significantly higher in HeLaCx43-EGFP than HeLa parental cells.

fluorescence intensity represent clustering of Cx43-EGFP channels into GJs or JPs (see white arrows). Here, and as previously shown [43], functional cell–cell coupling is proportional to number and size of junctional plaques. Often, fluorescent vesicles are present in the cytoplasm and represent internalized junctional plaques (see yellow arrowheads). Figure 1B shows the percentage of cells distributed among five categories (see Section 2) based on AO and EB staining measured after the cells were treated with anisomycin (1 μg/mL) for 3, 6, 16, 24, 48, and 72 hr. The data show that although departure from a viable non-apoptotic state occurs with similar kinetics in HeLa parental and HeLaCx43-EGFP cells over a 72 hr period, the percentage of cells in the NVA state was substantially higher in the HeLaCx43-EGFP transfectants as early as 6 hr following treatment with anisomycin. The increase in the percentage of HeLaCx43-EGFP cells in the NVA state was accompanied by a decrease in the VA state suggesting that Cx43-EGFP expression may speed transition of apoptotic cells from the VA to the NVA state.

To determine whether the accelerated transition to the NVA state was specific to anisomycin, we examined the effect of a variety of agents known to induce apoptosis. Figure 2 shows the effects 48 hr after treatment. Although all of the apoptotic agents decreased the percentage of viable cells and increased the percentage of apoptotic cells to different degrees, in all cases HeLaCx43-EGFP cells exhibited a higher percentage of cells in the NVA state compared to the HeLa parental cells. The percentages of necrotic and chromatin-free cells were low and showed no statistically significant differences among HeLa parental and HeLaCx43-EGFP cells.

3.2. Development of apoptosis and functional coupling

To examine whether the effect of Cx expression on the development of apoptosis was Cx specific and required functional cell–cell coupling, we performed three experiments. First, we examined whether attachment of EGFP to the C-terminus of Cx43 had any influence on the accelerated transitioning of HeLa cells into to the NVA state. Using two reagents, CAM and *cis*-platinum, no differences were found compared to cells transfected with Cx43-EGFP (Fig. 3A). Second, we examined whether accelerated transitioning of HeLaCx43-EGFP cells into the NVA state was blocked by adding a GJ channel blocker, octanol. Addition of octanol (0.1 mM) to cells treated with anisomycin (1 μg/mL) reduced the percentage of NVA cells, diminishing the difference between HeLa parental and HeLaCx43-EGFP cells (Fig. 3B). Third, we examined HeLa cell lines stably transfected with Cx32-EGFP, the C-terminal fusion protein, and EGFP-Cx32, the N-terminal fusion protein. Previously we showed that cells expressing EGFP-Cx32 and EGFP-Cx43 form junctional plaques indistinguishable from those formed with Cx32-EGFP or Cx43-EGFP, respectively, but do not become functionally

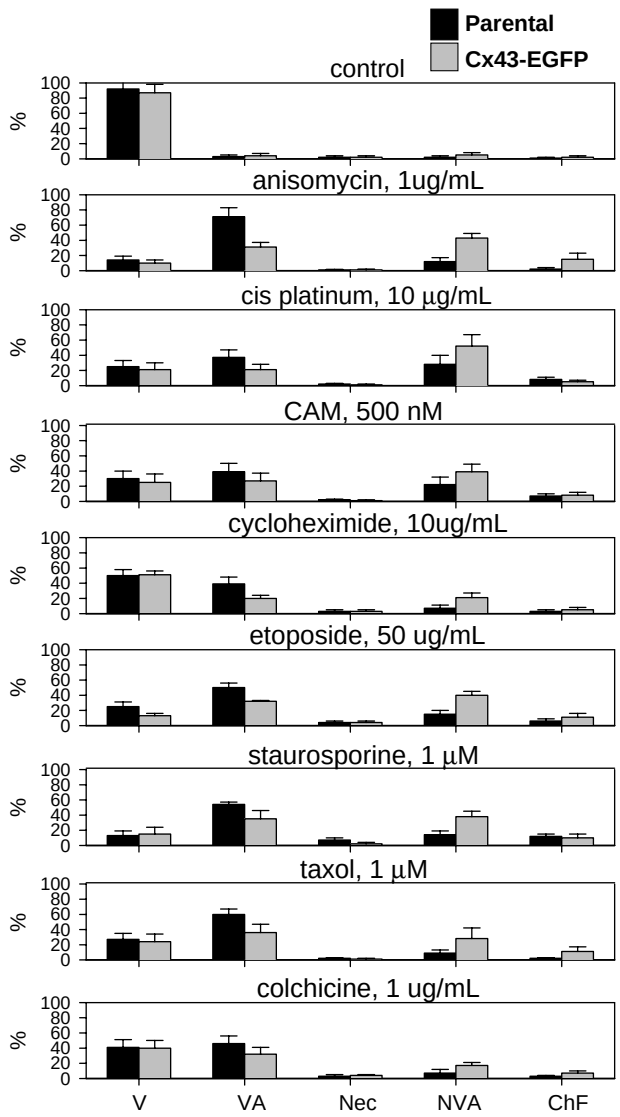

Fig. 2. HeLaCx43-EGFP cells (gray bars) show a higher percentage of NVA cells than HeLa parental cells (black bars) in response to a variety of agents that induce apoptosis. Apoptotic features were examined 48 hr following treatment. Shown are the distributions (in %) of cells evaluated with AO and EB fluorescence staining.

coupled [44]. We found that Cx32 N-terminal transfectants exhibited substantially fewer cells in the NVA state than Cx32 C-terminal transfectants (Fig. 3C), comparable to that observed in HeLa parental cells (compare VA and NVA bars of Fig. 3C with Figs. 1B and 2). These data support the octanol data and show that functional cell–cell coupling, and not just Cx assembly into plaques, is essential for accelerating the transition from VA to NVA state. In addition, these data demonstrate that the effect is not specific to Cx43 or Cx43-EGFP as functional Cx32-EGFP expression gives similar results.

3.3. Cx43-EGFP hemichannels

Some Cxs have been shown to be capable of functioning as hemichannels in the plasma membrane of cells [45–48].

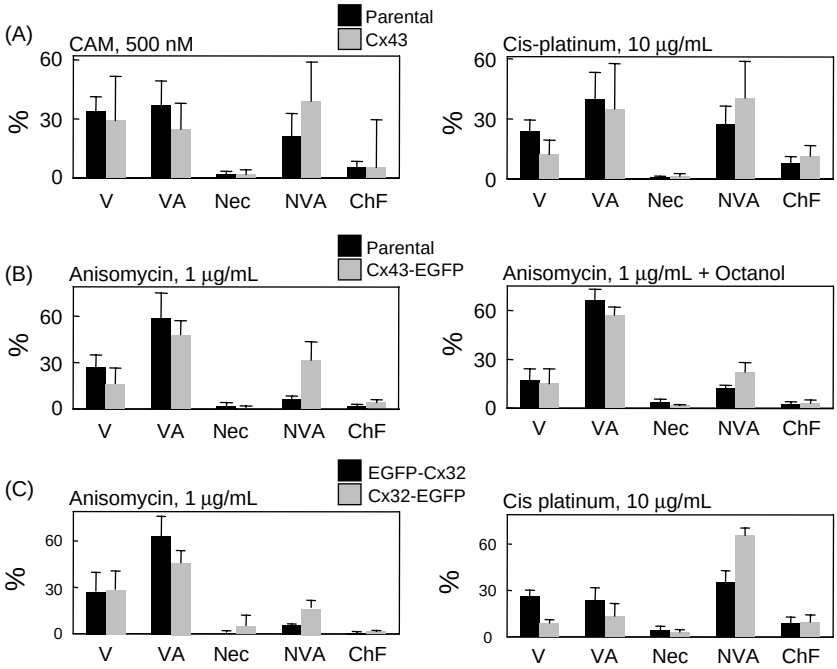

Fig. 3. (A) Response of HeLa cells expressing wild-type Cx43 is the same as those expressing Cx43-EGFP. The apoptotic agents used were CAM and *cis*-platinum at the concentrations indicated. Cells were examined after 48 hr of treatment. Black and gray bars correspond to HeLa parental cells and HeLa cells expressing Cx43, respectively. (B) The GJ channel blocker, octanol (0.1 mM), reduces the effect of Cx43-EGFP on the transition from the VA to the NVA state. Cells were exposed to octanol 30 min before treatment with anisomycin. The cells were assessed 20 hr after treatment with anisomycin alone (top panel) or together with octanol (bottom panel). Black and gray bars correspond to HeLa parental cells and HeLa cells expressing Cx43-EGFP, respectively. (C) N-terminal EGFP connexin Cx32 transfectants, which form plaques indistinguishable form C-terminal EGFP transfectants, but fail to provide functional coupling, do not show the accelerated transition from the VA to the NVA state. Black and gray bars correspond to HeLa cells expressing EGFP-Cx32 and Cx32-EGFP, respectively.

To examine whether Cx43-EGFP hemichannels were functional in our HeLa Cx43-EGFP transfectants, we examined differences in long-term dye uptake between HeLa parental and HeLaCx43-EGFP cells. Such differences have been shown to accompany hemichannel opening in the plasma membrane [48]. These experiments were accomplished using time-lapse imaging of two fluorophores, EGFP and EB, in co-cultures of HeLa parental and HeLaCx43-EGFP cells. HeLaCx43-EGFP cells can be easily identified by EGFP fluorescence and junctional plaques are strong indicators of functional coupling. Figure 4A and B show averaged (each curve is averaged from four experiments; in each experiment fluorescence intensity is averaged from 10 randomly selected cells) and normalized plots of fluorescence intensity for untreated cells (A) and cells treated with anisomycin (B). In the untreated cells, EB fluorescence increased almost linearly over time both in HeLaCx43-EGFP (open circles) and HeLa parental (filled circles) cells. However, fluorescence intensity was nearly 2-fold greater in HeLaCx43-EGFP than in HeLa parental cells consistent with more dye uptake in the presence of functional Cx43-EGFP hemichannels. Treatment with anisomycin strongly suppressed dye uptake in both cell types, but more so in cells expressing Cx43-EGFP. Interestingly, during the initial period of anisomycin treatment (0–3 hr), dye uptake appeared similar to control conditions (no treatment) and then fell to an apparent steady-state value suggesting that dye uptake was completely blocked after 15 hr of treatment.

3.4. Connexin expression and junctional plaques

To examine whether agents that induce apoptosis affect Cx distribution, we determined whether there were any changes in total Cx43-EGFP fluorescence as well as in the number of junctional plaques among contacting cells. In these experiments apoptotic agents were applied to 1-day old confluent cell cultures of HeLaCx43-EGFP cells. Figure 4C shows the time course of total Cx43-EGFP fluorescence measured at different time intervals up to 48 hr in HeLaCx43-EGFP cells untreated (control) and treated with different apoptotic agents. The total EGFP fluorescence was studied by using UltraView software, which allows measurement of the time course of fluorescence in selected regions of interest as well as elimination of background fluorescence. Averaged and normalized data show that total Cx43-EGFP fluorescence decreases ~2-fold after 48 hr of treatment with apoptotic agents. The numbers of JPs were assessed in the same experiments and counted in randomly selected regions of interest (~100 µm × 100 µm). All data were normalized to the number of JPs measured at the start of each experiment.

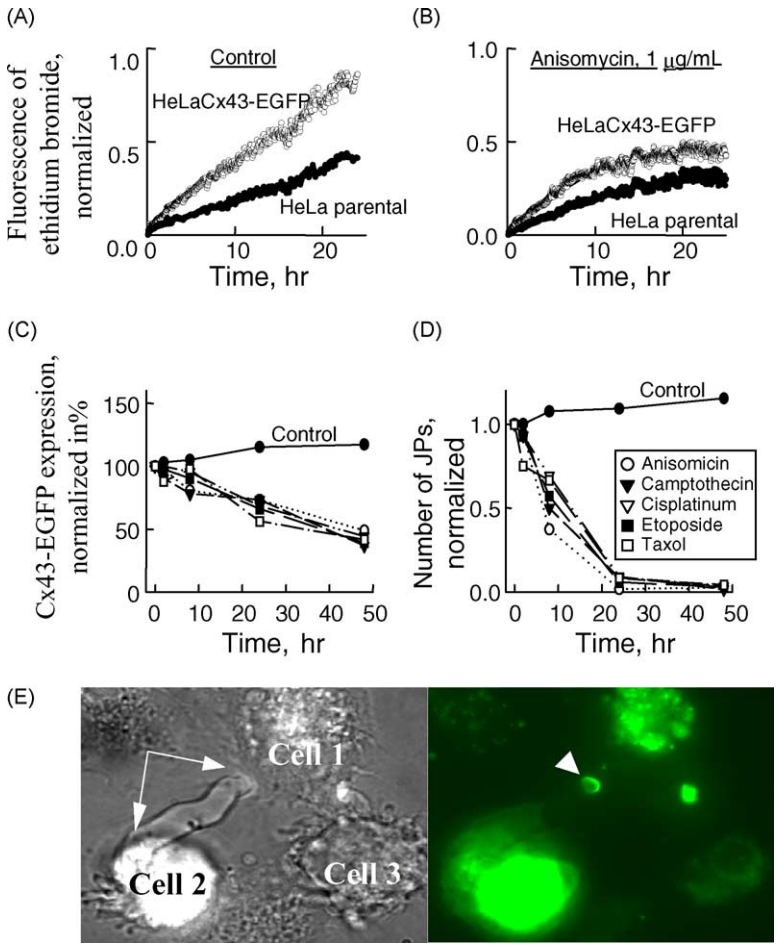

Fig. 4. (A, B) Anisomycin inhibits EB uptake through Cx43-EGFP hemichannels. (A) EB uptake over time measured as normalized fluorescence intensity in HeLaCx43-EGFP (open circles) and HeLa parental (filed circles) cells; cells were incubated (5% CO_2 and 37°) and perfused with DMEM containing 5 μM EB. The experiments were performed in co-cultures of HeLa parental and HeLaCx43-EGFP cells. Petri dishes with thin glass bottoms containing the co-cultures were positioned into a micro-incubator adapted onto the stage of inverted microscope. Time-lapse imaging started just before addition of EB (control) to the perfusion medium. Excitation and emission wavelengths of EGFP and EB do not overlap. (B) The same as in (A), but in addition perfusion medium contained anisomycin, 1 μg/mL. (C, D) Changes in Cx43-EGFP expression and distribution after treatment with apoptotic agents. Time-lapse imaging was used to evaluate changes in total fluorescence and the number of junctional plaques in HeLaCx43-EGFP cells incubated (5% CO_2 and 37°) on the stage of an inverted microscope for 48 hr. (C) Changes in total EGFP fluorescence. Each trace is averaged from measurements performed in four experiments for each apoptotic agent; in each experiment fluorescence intensity was measured from six randomly selected fields of view. (D) Changes in the number of Cx43-EGFP junctional plaques. Junctional plaques were assessed in the same experiments and the same fields of view as in (C). The data are averaged and normalized to the data values measured at the beginning of the experiments. (E) Phase-contrast (left) and fluorescence (right) images of HeLaCx43-EGFP cells 16 hr after treatment with anisomycin. HeLaCx43-EGFP cells undergoing apoptosis maintain contact through long cytoplasmic extensions (tube-like structure between cells 1 and 2 shown by two arrows in the phase contrast image). In the example shown, a junctional plaque remains within the cytoplasmic extensions (arrowhead in the fluorescence image).

Our data indicate that the number of JPs decays much faster than the total amount of Cx43-EGFP fluorescence, reaching almost zero after 48 hr (Fig. 4D).

Time-lapse imaging showed that during apoptotic transformations, junctional plaques were progressively internalized by endocytosis as evidenced by the formation of cytoplasm vesicles. Junctional plaques that were not rapidly internalized caused cells to form long cytoplasm extensions as the cells shrank away from each other (see phase contrast image and arrow in Fig. 4E). The formation of these cytoplasm extensions suggests that the interaction between hemichannels in apposing cells is strong and that junctional plaques can withstand substantial stresses resulting from cellular deformation and movement. The remaining plaques were functional as assessed by the dual whole-cell patch clamp method (see below).

3.5. Intracellular ATP

Studies in C6 glioma, HeLa, and U373 glioblastoma cells reported a substantial increase in ATP release after transfection with Cx43 [49]. Thus, we compared ATP concentrations in HeLa parental and HeLaCx43-EGFP cells with and without anisomycin treatment. In the absence of anisomycin, we found ATP levels in HeLaCx43-EGFP cells to be ~4-fold lower than in HeLa parental cells (Fig. 5A). This can be explained by the loss of ATP through Cx43-EGFP hemichannels. Although ATP concentration was lower in

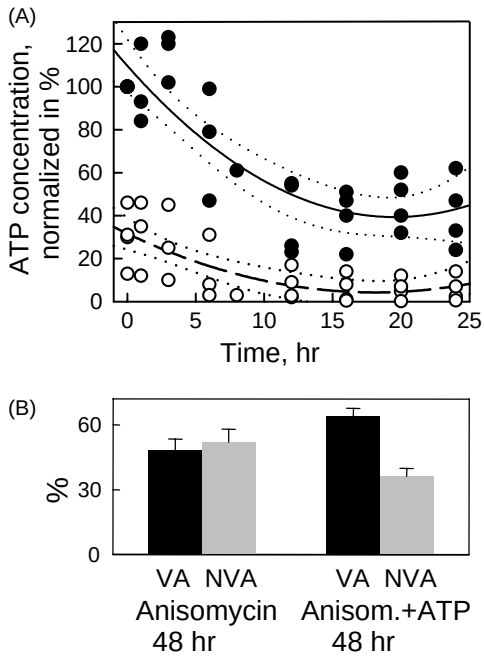

Fig. 5. (A) Time course of the change in intracellular ATP in HeLa parental cells (filled circles) and HeLaCx43-EGFP cells (open circles) in the presence of anisomycin (1 μg/mL) added to perfusion medium. ATP concentration is normalized (in %) to the concentration in HeLa parental cells measured under control conditions. Solid and dashed lines are regression curves determined in four experiments, respectively, for HeLa parental and HeLaCx43-EGFP cells; dotted lines show 95% confidential intervals. (B) ATP (0.1 mM) added into perfusion medium reduces cells' transition from a VA (black bars) to a NVA (gray bars) state. HeLaCx43-EGFP cells were treated with anisomycin alone or together with ATP for 48 hr.

HeLaCx43-EGFP cells, both cell types exhibited a nearly proportional decline in ATP concentration during 24 hr of treatment with anisomycin. Despite the difference in ATP concentration between HeLa parental and HeLaCx43-EGFP cells, both cell lines appeared to enter apoptosis at the same time suggesting that the differences in ATP concentration are not critical for induction of apoptosis. However, the lower ATP concentration may be involved in speeding the transition from a VA into a NVA state.

Addition of ATP (0.1 mM) to the perfusion solution in the presence of anisomycin increased the ratio of VA vs. NVA from 0.97 ± 0.25 to 1.8 ± 0.37 (Student's t-test shows statistically significant difference; $T = 5.8$ and $P < 0.0001$) (Fig. 5B). These experiments show that ATP is critical for apoptotic transformations and extracellular ATP can modulate the dynamics of the development of apoptosis.

3.6. Electrical cell–cell coupling

We examined whether apoptotic agents, themselves, act as uncoupling factors during short and long-term treatments. Experiments were performed by using the dual whole-cell voltage clamp method (for details see [50]). For short-term studies, we examined isolated HeLaCx43-EGFP cell pairs containing at least one junctional plaque. After establishing a dual whole cell recording, we applied high concentrations of an apoptotic agent such as anisomycin (5 μg/mL), cycloheximide (50 μg/mL), staurosporine (5 μM) or etoposide (250 μM); three experiments for each examined compound. None of these agents caused measurable changes in junctional conductance (γ_j) during a ~30 min application. In each case heptanol (2 mM) or solution saturated with 100% CO_2, conditions that are well known to block gap junction channels [51], caused reversible uncoupling indicating that the cell pairs were coupled by GJs and not cytoplasmic bridges.

In addition, HeLaCx43-EGFP cells were incubated for ~24 hr in the presence of *cis*-platinum (1 μg/mL), CAM (0.5 μM), etoposide (50 μM), cycloheximide (10 μg/mL) or taxol (1 μM). During this time period, cells demonstrated typical apoptotic transformations. We examined coupling in cell pairs that exhibited at least one junctional plaque. Previously, we demonstrated that only cell pairs containing junctional plaque(s) show electrical cell–cell coupling [43]. In each of 11 cell pairs tested, the junctional plaques were located within the cytoplasmic extensions as previously described (Fig. 4E). All these cell pairs demonstrated electrical cell–cell coupling and responded to heptanol or cytoplasmic acidification by saturating the perfusion solution with 100% CO_2 by full uncoupling. Both, heptanol and CO_2, are known as ''classical'' uncouplers of gap junction channels [52,53]. In a cell pair treated with cycloheximide for 24 hr, recording of single channels showed a conductance, γ_j, of 120 pS, the same as untreated Cx43-EGFP channels [50] indicating that apoptotic agents do not affect GJ channel unitary conductance.

4. Discussion

Although there is a large body of evidence that GJs are implicated in the regulation of cell growth, as well as in tumor suppression [54–56], the role of GJs in apoptosis has remained uncertain. Propagation of programmed cell death through GJs has been reported in several model systems *in vitro*. The capacity of cells to kill each other through GJs has been shown in ''bystander death'' experiments, where toxin spreads via GJs from affected cells into neighboring unaffected cells and eventually kills them [57,58]. GJ-mediated propagation and amplification of cell injury was shown in astrocytes after ischemic injury [27,48]. Krutovskikh *et al.* [28] demonstrated that bladder carcinoma cells (cell line BC31), which spontaneously die through apoptosis and are coupled with non-apoptotic counterparts, spread killing signals to their neighbors. It was suggested that Ca^{2+} ions are messengers that transfer cell-killing signals through GJs. However, Ando *et al.* [59] demonstrated that neither the time course of the morphological changes nor the time interval between the beginning of

apoptosis and secondary necrosis were related to the presence of calcium in the medium. Huang *et al.* [32] reported that Cx43 expression facilitates apoptotic transformation of human glioblastoma cells, and it was suggested that the Cx43-mediated apoptosis was executed, at least in part, through the downregulation of Bcl-2 (specific apoptosis-inhibitor) expression. Moreover, the anti-apoptotic effect of Cx43 expression was demonstrated in mouse embryonic fibroblasts treated with bisphosphonates and it was suggested that Cx43 hemichannels may be involved in transduction of anti-apoptotic signals [29].

4.1. Connexin expression and dynamics of the development of apoptosis

In this study, we examined apoptosis induced by a variety of apoptotic agents acting on different intracellular targets and whether Cx expression had any effect. The results of our studies show that although the extent and time course of apoptosis was specific to the inductive reagent used, the initiation of cell death as well as its progression, estimated by the nuclear changes in the population of parental cells and in cells transfected with Cx43-EGFP, did not differ greatly. The percentages of viable non-apoptotic cells with intact nuclei and non-leaky membranes were the same in both populations of cells over the time interval tested (Fig. 1B). The apoptotic cells with signs of nuclear condensation and segmentation appeared at the same time after induction and continued to increase during an extended period after apoptotic treatments. However, there was a faster progression of VA cells into a NVA state characterized by permeabilized membranes in HeLa cells transfected with Cxs. Thus, it appears that connexin expression does not influence the transition from a viable non-apoptotic to a VA state, but accelerates the progression of apoptotic cells from a VA state to a NVA state. An accelerated progression into a NVA state occurred with all tested cell lines that were expressing connexins, including Cx43-EGFP, Cx43 and Cx32-EGFP. Transformation of cells into a VA state occurred concomitant with cell shrinkage, ‘‘rounding’’ and internalization of junctional plaques. However, junctional plaques were present and remained functional during this transformation suggesting that reduction in functional coupling during the progression of apoptosis is caused by removal of GJ channels and not by their functional closure. Studies of apoptotic cells have shown that most often apoptotic bodies are remarkably stable and retain their membranes relatively intact over a long period of time [60]. *In situ*, apoptotic bodies are quickly engulfed by macrophages or neighboring cells without eliciting an inflammatory response. Transformation of cells into a NVA state, also called secondary necrosis or apoptotic necrosis, is characterized by breakdown of the plasma membrane permeability barrier and may develop as a consequence of ATP depletion and mitochondrial failure [61,62].

4.2. Connexin redistribution during the development of apoptosis

We demonstrate that all the apoptotic agents caused significant changes in Cx distribution characterized by a decline in the total amount of Cx (assessed from EGFP fluorescence) and a decline in the number of junctional plaques through increased internalization. Junctional plaques represent clusters of GJ channels and are the anatomical correlates of functional coupling. Only cells that contain junctional plaques are electrically and metabolically coupled, and interestingly only a small fraction of GJ channels within a junctional plaque is functional [43]. Furthermore, there appears to be a critical number of channels within a junctional plaque that confers function to GJ channels [43]. Although there were differences among the different apoptotic agents used, with all of them we observed a progressive decline in the number of junctional plaques. On average, the number of plaques decreased by ~50% after a 12 hr treatment. We and others have reported that cells transfected with Cx-EGFP constructs [33,63] show junctional plaques to be highly dynamic structures, that their assembly is organized through fusion of hemichannel vesicles into the plasma membrane and that growth occurs by lateral attraction of hemichannels from adjacent regions of the plasma membrane [64,65]. During the development of apoptosis, cells shrink thereby forming cytoplasmic extensions in the regions where cells are in contact and contain junctional plaques. The formation of cytoplasmic extensions (see Fig. 4E) indicate that interactions between hemichannels across the extracellular gap are strong and that GJs remain intact despite forces working to pull cells apart. Our electrophysiological measurements using the dual whole-cell voltage clamp method demonstrate that junctional plaques within the cytoplasmic extensions still maintain functional coupling. Eventually, we observed internalization of these junctional plaques through the formation of endocytic vesicles and no new formation of junctional plaques [66]. Thus, we conclude that all of the apoptotic agents we used dramatically decreased the total amounts of Cx43-EGFP (see time course of Cx43-EGFP fluorescence decay in Fig. 4C), presumably, through inhibition of *de novo* synthesis of Cx43-EGFP as well as accelerated internalization of Cx43-EGFP junctional plaques (see Fig. 4D) followed by their degradation.

4.3. Functional cell–cell coupling and apoptosis development

Previously, it was reported that attachment of EGFP to the C terminus of Cx43 does not affect single channel conductance or selectivity properties assessed by cell–cell dye transfer [43]. Cx43 GJ channels have a unitary conductance of ~120 pS, allow both cations and anions to pass more or less equally [50] and are permeable to metabolites

with molecular weights up to ~1 kDa [53]. Our data indicate that short (~30 min) exposure to apoptotic agents even at concentrations ~5-fold higher than those used in our longer-term studies caused no changes in electrical coupling. We report that even 24 hr after perfusion with apoptotic agents, the few junctional plaques that remained were still functional, single Cx43-EGFP channel conductance remained unchanged and the channels retained their sensitivity to the uncouplers, heptanol and CO_2. These data indicate that the reduction in coupling we observed during the development of apoptosis was caused by a reduction in the number of GJ channels, evidenced by internalization of junctional plaques, and not by changes in GJ channel properties. Furthermore, maintenance of functional coupling appeared to be important in promoting the transition of cells to a NVA state. Blocking GJ channels with octanol (Fig. 3B) or using a GJ channel (EGFP-Cx32) that forms and clusters into plaques but does not function [44] eliminated the enhanced transformation of cells to a NVA state. GJ channel blockers have been shown to block hemichannels [48] and our unpublished data show that Cx43 and Cx43-EGFP form functional hemichannels, but that EGFP-Cx43 does not. Thus, use of GJ channel blockers, as well as use of N-terminal EGFP-Cx fusion proteins should block hemichannel function, as well as GJ channel function. Moreover, astrocytes cultured from Cx43 knockout animals demonstrate significantly lower level of dye uptake during inhibition of glycolytic and oxidative metabolism than astrocytes cultivated from normal animals [48]. Taken together, these data suggest that functional GJ channels and/or hemichannels are required for promoting secondary necrosis (NVA state) and that adhesive or other properties of GJ channels are not likely to play a significant role. Thus, it might be that intracellular factors are released during apoptosis that are important in accelerating the spread of secondary necrosis through a cell population, and that it occurs during the first 10–15 hr of apoptosis when a significant fraction of functional GJs are still present. This mechanism is similar to the ‘‘bystander effect’’ causing cell-to-cell transfer of ‘‘death’’ signals through GJ channels [57,58].

4.4. Connexin hemichannels and the development of apoptosis

There have been several studies reporting that Cx43 forms hemichannels that can open under normal conditions, albeit with low probability, and that open probability increases during metabolic inhibition [48]. Cx32 has also been reported to form functional hemichannels [67]. It was shown that Cx43 hemichannels are present in 3T3 fibroblasts and that they are permeable to NAD^+ [68]. Furthermore, it was shown that cells overexpressing Cx43 demonstrate over a ~10-fold increase of ATP release (‘secretion’) [49]. Given that GJ channel and hemichannel pore sizes are in the ~10–15 Å range [69,70], and that conductance of a hemichannel should be approximately twice that of a GJ channel (~$120 \times 2 = 240$ pS for Cx43), opening of such large, high conductance pores should increase membrane permeability, disturb ionic balance and cause leak of metabolites with molecular weights up to 1 kDa [53]. We examined hemichannel function by measuring EB uptake, as was done previously in astrocytes [48]. Under normal conditions, we show a nearly 2-fold increase in EB uptake in HeLaCx43-EGFP cells compared to HeLa parental cells (see Fig. 4A). This difference can be attributed to Cx43-EGFP hemichannel openings. We found that anisomycin decreased EB uptake and almost completely blocked it after 15 hr (see Fig. 4B). Our data are consistent with the reports that gap junction channel blockers, such as heptanol, octanol [71], 18α-glycirrhetinic acid [48,72] and flufenamic acid [73] inhibit dye uptake through connexin hemichannels.

Plasma membrane permeability ascribable to Cx43-EGFP hemichannels appears to decrease during the development of apoptosis. Since gating and susceptibility to blocking agents are properties conserved in GJ channels and hemichannels [46,48,74], the decrease in dye uptake, presumably is caused not only by an inhibition of hemichannel activity but also by a number of additional mechanisms including a decrease in Cx43-EGFP synthesis and its oligomerization into hemichannels, disruption of hemichannel vesicle transport to the plasma membrane due to disruption of the cytoskeleton, and enhanced removal of Cx43-EGFP hemichannels from the plasma membrane. It is well established that the half-life of wild-type Cxs [75–77] as well as Cxs fused with EGFP [63] is only several hours so that normal removal in the presence of blocked assembly or insertion can lead to rapid loss of hemichannels. During the development of apoptosis, when protein synthesis is strongly suppressed or fully blocked, the density of hemichannels in the plasma membrane should, in fact, decay rapidly. Although there is evidence suggesting that hemichannels may contribute to speeding the process of apoptosis, we cannot differentiate between the roles of hemichannels and GJ channels in this process.

4.5. ATP and apoptosis

Several reports have suggested that intracellular ATP is critical in transforming cells into apoptotic or necrotic states. Some level of ATP is required for nuclear condensation and DNA fragmentation, and when cells are strongly depleted of ATP they transform into a necrotic state [78,79]. However, the involvement of ATP levels in promoting secondary necrosis, i.e. via apoptosis, remains to be determined. Recently it was demonstrated that extracellular ATP can induce apoptosis in the neuroblastoma cell line (N1E-115) via ATP breakdown to adenosine [80]. Purinergic receptor-activated intracellular Ca^{2+} mobilization can induce a ~10-fold higher ATP release in cells transfected with Cx43 than in nontransfected cells suggesting a

role for Cxs in ATP depletion [49]. In support of this, Bruzzone *et al.* [68] demonstrated that Cx43-expressing 3T3 fibroblasts are permeable to NAD^+ and this permeability was completely inhibited by treatment with Cx43-antisense oligonucleotides. These authors concluded that Cx43 can form functional hemichannels in the cell surface and that they are highly permeable to metabolites. A similar conclusion was made based on enhanced dye uptake with metabolic inhibition of astrocytes expressing Cx43 [48]. Our data show that HelaCx43-EGFP cells have a substantially lower concentration of ATP than HeLa parental cells under basal conditions (see ATP concentration at 0 hr in Fig. 5A). We suggest that Cx43-EGFP hemichannels may be responsible for this difference. Under the effect of agents that induce apoptosis, ATP concentration declined proportionally in both, HeLa parental cells and HeLa cells transfected with Cx43-EGFP, but ATP levels were significantly lower in HeLaCx43-EGFP cells (Fig. 5A). Interestingly, extracellular ATP (100 μM) partially rescues HeLaCx43-EGFP cells from secondary necrosis (Fig. 5B). We suggest that Cx hemichannels can open which may lead to an imbalance of ionic gradients and a loss of some essential metabolites, including ATP [68].

These studies demonstrate that Cxs appear to modulate the development of apoptosis through their abilities to form functional hemichannels and GJ channels. Numerous reports demonstrating the importance of Cxs in development or malignant cell growth when cells are actively proliferating [81–84] suggest that Cxs may represent a key target point for modulation of apoptosis with possible therapeutic implications. Pharmacological modulation of cellular communication in a positive or negative direction can provide a novel means to combat, neurodegenerative disorders such as Alzheimer disease [85], atherosclerosis [86], etc., that involve apoptosis. We show that Cxs are active players in the spread of apoptosis and are likely to act by transferring apoptotic signals from cell-to-cell through GJ channels and/or hemichannels.

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