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The spread of apoptosis through gap-junctional channels in BHK cells transfected with Cx32

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Programmed cell death (apoptosis) occurs both during normal development and as a result of various pathological conditions. An *in vitro* system was used to explore the transmission of death signals from apoptotic cells to cells with which they were coupled via gap junctions. Confluent cultures of baby hamster kidney (BHK) cells, stably transfected with the gap-junctional protein connexin32, were scrape loaded with cytochrome C (cyC), a mitochondria-derived apoptotic agent, to introduce the protein into cells injured by the cut. The cultures were subsequently analyzed for the presence of activated caspases, the distribution of TUNEL staining, and the binding of annexin V. Although cyC is too large to traverse the gap junctional channel, each of the assays revealed that apoptosis had spread from dying cells at the margin of the scrape to otherwise healthy neighboring cells to which they were coupled. This 'bystander effect' was significantly reduced in the presence of agents that block gap junctional intercellular communication.

Keywords: annexin V; apoptosis; BHK cells; bystander cell death; caspase activity; connexin32; cytochrome C; gap junction; intercellular communication; transfection; TUNEL staining.

Introduction

Gap junctional intercellular communication (GJIC) is mediated by a multigene family of trans-membrane proteins called connexins.[1] A radial array of six connexin molecules assemble to form a connexon (hemichannel) which docks with its counterpart on a neighboring cell to form an intercellular pore ($d \approx 16$Å) that allows the rapid exchange of ions, metabolites and other small molecules having a molecular mass ≤ 1 kD. Connexins are expressed ubiquitously throughout the body, and gap junctions are essential to many vital processes, including growth, differentiation, homeostasis, and the coordination of cellular activities within the heart, brain, and other organs. In addition to these normative functions, there is mounting evidence that gap junctions are involved in the intercellular spread of apoptotic cell death both during development and in pathological conditions.

The propagation of apoptosis from cells that are destined to die to their otherwise healthy neighbors is commonly referred to as the "bystander effect", a phenomenon that has been observed in the transfer of toxic metabolites from tumor cells targeted for chemically-induced death to neighboring untreated cells.[2–4] A form of bystander cell death has also been reported in glia following cerebral ischemia,[5] and in the clustering of apoptotic cells during the course of retinal development.[6,7] In each of these situations, gap junctions have been implicated in the intercellular transmission of agents that induce apoptosis, although neither the connexin(s) mediating the transfer, nor the nature of the noxious agent transmitted intercellularly, could be identified.

In the present study we used an *in vitro* system, in which cytochrome C was used to induce apoptosis in a cell line expressing Cx32, and thereby test whether gap junctions formed by a well-characterized connexin are able to mediate bystander cell death. The results provide strong evidence in support of this view. Cytochrome C, a major electron transport protein of the inner mitochondrial membrane, is involved in oxidative phosphorylation, the principal source of ATP in aerobic organisms. However, opening of the mitochondrial transition pore, and translocation of cytochrome C to the cytosol, activates a cascade of enzymatic reactions that lead to cell death (see Discussion). Thus, introducing cytochrome C intracellularly, either by scrape loading or microinjection, provides a useful tool for the study of apoptosis. We found that after scrape loading cytochrome C (cyC) into a cluster of confluent baby hamster kidney (BHK) cells stably transfected with human connexin32 (hCx32), cell death extended from the cells incorporating this apoptosis-inducing agent to coupled cells far removed from the scrape. Wild-type BHK cells, not transfected with the connexin did not form gap junctions, nor did they exhibit bystander cell death. In addition, blockers of GJIC such as carbenoxolone

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and octanol were effective in preventing the spread of apoptosis.

Materials and methods

Reagents

The sources from which we obtained PCR products and primary and secondary antibodies are indicated in the text; all other chemicals were analytical grade or better, and purchased from Sigma-Aldrich, St. Louis, MO.

Cell cultures

BHK cells stably transfected with human connexin32 DNA were derived from stock cultures prepared as described previously.[8] The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin, and kept in a 37°C incubator under a humidified 5% CO_2/95% air atmosphere. Cells were dissociated twice weekly by mild treatment with trypsin/EDTA and subcultured in the growth medium.

Connexin32 expression

To ensure that the transfected BHK cells expressed Cx32, the cultures were examined by immunocytochemistry, Western blot analysis, and RT-PCR. For immunochemistry, cells were plated on sterile 35 mm plastic dishes, grown to near confluency, washed with PBS, and fixed with 4% paraformaldehyde in PBS. They were then blocked in a solution containing 3% fetal calf serum and 0.3% Triton-X-100 in PBS, and incubated at 4°C with a mouse monoclonal connexin32 antibody (Chemicon, Temecula, CA) diluted 1:500 in the blocking medium for 48 hours. After washing three times with the blocking solution, cells were incubated for ~30 minutes with a goat anti-mouse secondary antibody tagged with Cy3 (Jackson ImmunoResearch Laboratories, West Grove, PA), washed with PBS, and a few drops of Vectashield were added.

For Western blots, BHK cells were harvested by trypsinization, and 100 μl of a membrane protein lysis buffer added to the cell pellet; BHK cells not transfected with connexin32 were used as the negative control. After homogenization, 20 μl of denaturing gel loading buffer was added and the mixture incubated at 95°C for 5 minutes. The protein extracts were run on a 12%Tris-HCL SDS-polyacrylamide gel (BioRad Laboratories, Hercules, CA) and samples were transferred to a nitrocellulose membrane in a BioRad TransBlot overnight. The membrane was blocked in a solution containing 3% milk powder in TBST buffer for 1 hour, and incubated with the mouse monoclonal Cx32 antibody. A SuperSignal West Pico Complete Mouse IgG Detection Kit (Pierce, Rockford. IL) was used according to the manufacturer's directions for incorporating the HRP conjugated goat anti-mouse IgG, and the tagged protein-antibody complex was detected by chemiluminescence after exposure to autoradiographic film.

RT-PCR was performed on cells harvested with Trizol (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's protocol for extracting small amounts of RNA. RT-PCR was performed with a one-step RT-PCR kit (Qiagen Inc., Valencia, CA) according to the manufactures instructions with gene-specific primers (sense: 5′-GTGGTGTTCCGGCTGTTGTT-3′; antisense: 5′-GGCCACATTGAGGATGATGC-3′) for human connexin32 (Integrated DNA Technologies, Coralville, IA). The PCR products were run on a 2% agarose gel and stained with ethidium bromide.

Scrape loading, dye transfer, and induction of apoptosis

The use of the scrape-loading dye-transfer technique introduced by El-Fouly *et al*.[9] has proven to be a reliable method for the study of GJIC. Although difficulties have been encountered in the quantitative assessment of results derived from this approach,[10] image analysis methods[11] fulfill the need for a rapid, sensitive determination of GJIC[12] and the spread of cell death.[7]

To determine whether the BHK cells expressing Cx32 formed gap junctions with their neighbors, the cells were grown to near confluency in 35 mm plastic dishes, washed with PBS, and then bathed in 150 μl of a PBS solution containing 0.05% Lucifer yellow and 1% tetramethylrhodamine dextran (Molecular Probes, Eugene OR). Lucifer yellow (457 D) readily passes through gap junctions, whereas the dextran complex (10 kD) does not.[13] A region of the monolayer, viewed under a stereomicroscope, was gently cut with a fine scalpel blade, and the cells were incubated in the solution for 5 minutes to allow the dyes to infiltrate the cut cells. After several washes to exchange the extracellular dye solution for PBS, the cells were fixed with 4% paraformaldehyde and examined with a Zeiss Axiovert 100 M fluorescent microscope (Carl Zeiss, Oberkochen, Germany).

Induction of apoptosis by cytochrome C: Cells were grown as before in 35 mm plastic dishes, or alternatively on 12 mm poly-D-lysine coated cover slips (Fisher Scientific, Hanover Park, IL). The growth medium was removed by pipetting, and the cells were immersed in a PBS solution containing 100 μl of 1 mM cytochrome C (cyC). A small cut was made in the monolayer, and the cells were incubated for 2 min in the cyC solution at room temperature.

Growth medium was then added to the cyC solution and the cells incubated in the mixture for 1 hr at 37°C. After repeated rinses in PBS to remove the cyC, the cells were analyzed for the presence of various apoptotic markers. To ensure that the spread of apoptosis was mediated by gap junctions, control experiments were conducted by introducing gap-junction blockers, 1 mM octanol or 800 μM carbenoxolone (18 β-glycyrrhetinic acid), 5 min before the addition of cyC, and through the use of communication deficient wild-type BHK cells as a negative control. Cells that took up the cyC were identified by immunocytochemistry, utilizing a mouse monoclonal primary antibody to cyC (Santa Cruz Biotechnology, Santa Cruz, CA). In several experiments, the tetramethylrhodamine dextran conjugate was scrape loaded together with cyC; since neither cyC nor dextran can traverse gap junctions, incorporation of both agents served as useful indices of those cells opened to the extracellular milieu by the scrape.

Cell death assays

TUNEL staining: DNA degradation is considered to be a major indicator of apoptosis. In the TUNEL assay, partially degraded DNA is labeled with fluorescein-dUTP by using terminal deoxynucleotidyltransferase (TdT) to introduce the nucleotide preferentially into 3′ strand breaks.[14] TUNEL label, a nucleotide mix containing fluorescein-dUTP and dNTP was obtained from Roche Applied Science (Indianapolis, IN). After induction of apoptosis by cyC, the cells were washed three times with PBS and fixed with 4% paraformaldehyde in PBS for 1 hour at room temperature. They were then rinsed with PBS, incubated for 2 min with an ice cold permeabilization solution containing 0.1% Triton X-100 in 0.1% sodium citrate, and rinsed again with PBS. 5 μl of TdT (Fermentas Inc., Hanover, MD) and 45 μl of TUNEL label solution were mixed well and added to the cells, which were allowed to incubate at 37°C for 1 hour in a closed humidified chamber. After two rinses in PBS, the cells were mounted in Vectashield and viewed under the fluorescent microscope.

Annexin V assay: Phosphatidylserine (PS), located on the inner leaflet of the cell membrane in normal living cells, is translocated to the outer leaflet as a result of the membrane reorganization that occurs soon after the induction of the apoptotic cascade.[15] Annexin V binds to PS with high affinity in the presence of calcium, and thus fluorescein conjugated annexin V serves as a marker for early stages of apoptosis.[16,17] An apoptosis detection kit containing FITC tagged annexin V was obtained from Oncogene Research Products (EMD Biosciences Inc., San Diego, CA), and the manufacturer's protocol modified for *in situ* analysis. Accordingly, after incubating the cells for 15 min in the annexin-FITC at room temperature in the dark, 0.5 ml of cold 1X binding buffer was added, the samples were placed on ice, and subsequently viewed by fluorescent microscopy.

Caspase activity: The family of cysteine-dependent aspartate-directed proteases (caspases) play a prominent role in apoptosis.[18] Once activated, they disrupt structural elements of the cytoplasm and nucleus, and cleave intracellular polypeptides and other substrates as cells undergo apoptosis. Immunocytochemistry for the presence of caspase activity was performed using rabbit polyclonal antibodies against cleaved caspases 3, 6, and 9, and a mouse monoclonal antibody against cleaved caspase 8 (Cell Signaling Technology, Beverly, MA). Cells were grown to near confluency and apoptosis induced as before by scrape loading cyC. The cells were washed with PBS, fixed with 4% paraformaldehyde, and immunocytochemistry was performed as described above. Except for caspase 8, for which a mouse monoclonal antibody was used, cells examined for caspase activity were double-stained with a mouse monoclonal antibody for cyC; caspase antibodies were diluted 1:100 and cyC antibody 1:200. The secondary antibodies were donkey anti-rabbit cy3 and goat anti-mouse FITC, respectively.

For each of the assays indicated, we determined the number of cells staining for a specific marker within a 9.6×10^5 μm^2 field that included the site of the scrape. Computing the ratio of stained cells to the entire cell population within the field gave the percentage values indicated by the histograms in each of the figures.

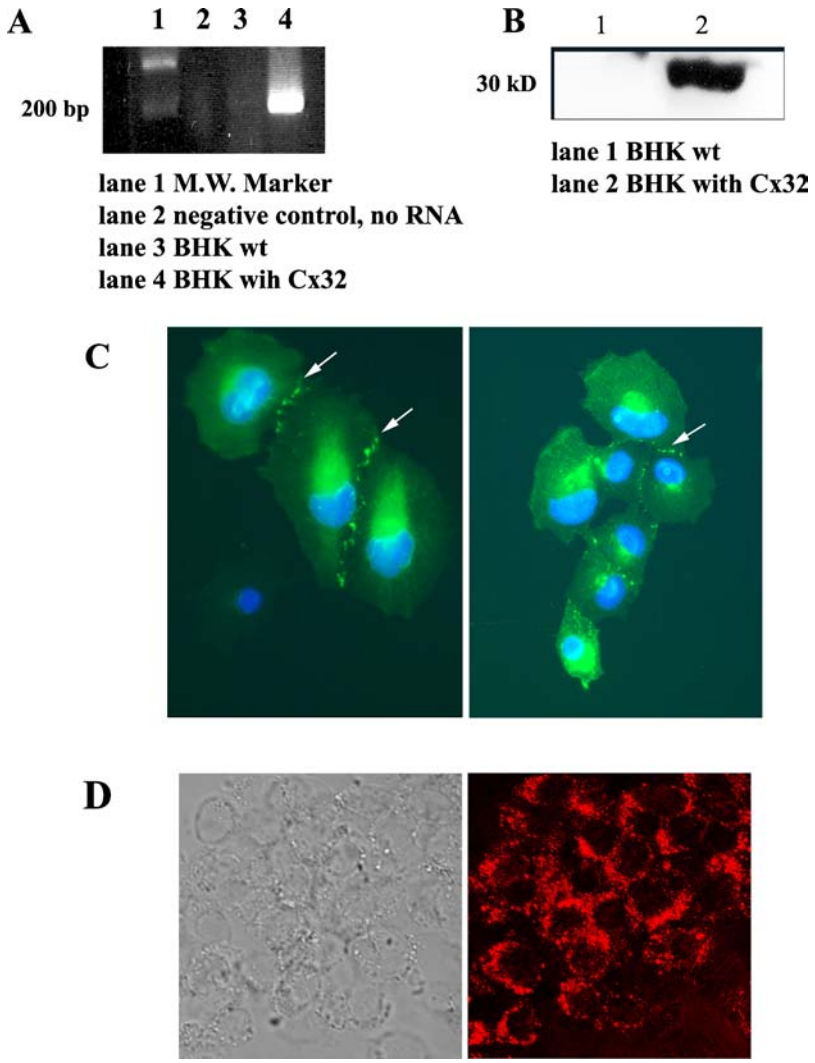
Image acquisition and processing

Cells examined for dye transfer and immunocytochemistry were visualized in a Zeiss Axiovert 100 M microscope (Zeiss, Oberkochen, Germany) through either a plan-Neofluar 20X/0.5 or an Achroplan 40X/0.6 phase objective (Zeiss), and photographed with a SensiCam CCD camera (resolution 1280 × 1024, Cooke Corp., Auburn Hills, MI). Image processing was controlled by MetaMorph software (Universal Imaging Corp., Westchester, PA).

Results

Cx32 expression: Transfected BHK cells expressed high levels of connexin32, as seen by immunocytochemistry, RT-PCR, and Western blot analysis. The RT-PCR results of Figure 1A show an intense band at ~200 bp (lane 4), the expected size for the Cx32 mRNA, whereas no signal for Cx32 mRNA was detected in the RT-PCR results from BHK cells not transfected with connexin32 (Figure 1A, lane 3). Similarly, a band at about 30 kD seen in Western blots of transfected cells (Figure 1B) was not evident in the

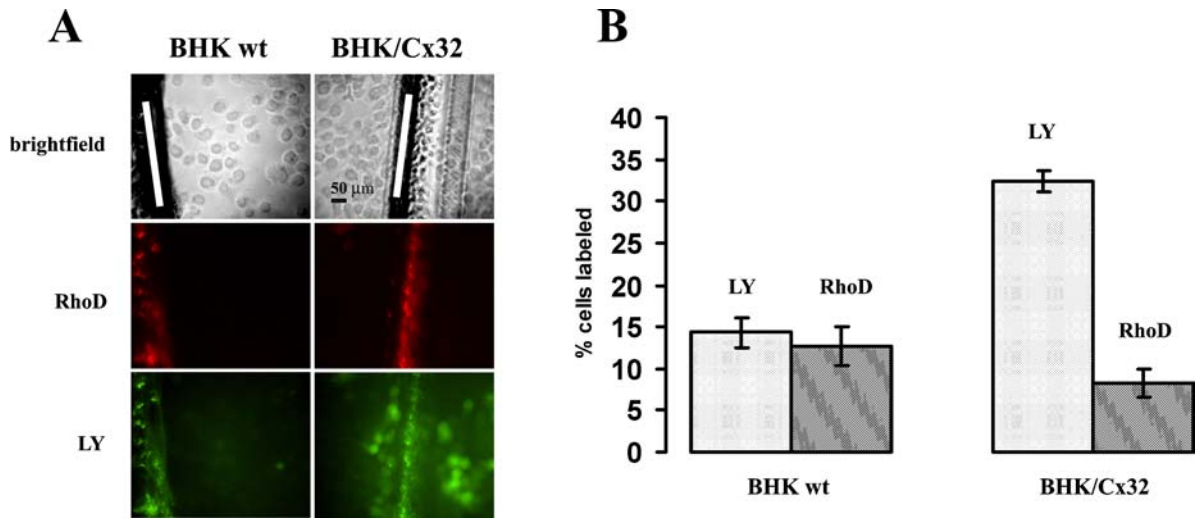
Figure 1. Expression of connexin32 in stably transfected BHK cells. (A) RT-PCR: ethidium bromide stained PCR products from wild-type BHK cells and cells transfected with Cx32. An intense band at ~200 bp, the expected size of the Cx32 mRNA, is seen only in lane 4, derived from Cx32 transfected cells. (B) Western blot: Autoradiographic image of chemiluminescent BHK cells transfected with Cx32 and incubated with the mouse monoclonal Cx32 antibody show a dense band at about 30 kD (lane 2); Western blots from wild type BHK cells not transfected with connexin32 served as the negative control (lane 1). (C) Early plaque formation (arrows) at the intercellular junctions of small groups of recently plated cells visualized with a mouse monoclonal antibody to Cx32 tagged with a FITC secondary in BHK cells whose nuclei are stained with DAPI. (D) The extensive distribution of Cx32 and multiple plaque formation seen on the membranes of confluent BHK cells labeled with the mouse monoclonal antibody to Cx32 tagged with Cy3; a bright field image is shown at the left.



lane carrying wild type BHK cells. Figure 1C shows early plaque formation at the intercellular junctions of small clusters of recently plated BHK cells visualized with a mouse monoclonal antibody to Cx32 tagged with a FITC secondary; cell nuclei were stained with DAPI. Figure 1D, in which confluent BHK cells were reacted with a Cx32 antibody and tagged with cy-3 (in red) show multiple plaque formation and the widespread distribution of connexins on the cell membranes. In contrast, wt-BHK cells did not react to the antibody (results not shown). Although Cx43 has been detected in wt-BHK cells, they are essentially communication deficient since the endogenous connexin appears to be trapped in the Golgi.[8] Thus, the intercellular gap junctional channels present in transfected BHK cells are formed almost exclusively by the expression of connexin32.

Cell coupling revealed by dye transfer: Cell coupling via gap junctions was visualized by comparing the intercellular transfer of Lucifer yellow (mw = 457 D) with that of tetramethylrhodamine dextran (mw = 10 kD) in wild type and Cx32 transfected BHK cells scrape loaded with both dyes (Figure 2). The results for non-transfected cells (BHK wt), illustrated in the left column of images in Figure 2A, show that cells along the scrape (indicated

Figure 2. Dye transfer assay with Lucifer yellow (LY) and tetramethylrhodamine dextran (RhoD). (A) BHK cells expressing Cx32 and scrape loaded with both fluorescent dyes demonstrate spread of LY to cells coupled through gap junctions, whereas BHK wt cells do not. The larger rhodamine-dextran molecule remains confined to cells injured by the scrape. The white line in each figure denotes the location of the scrape. (B) Histograms showing the percentage of cells labeled for the two dyes in wt and Cx32-transfected BHK cells. See text for details.

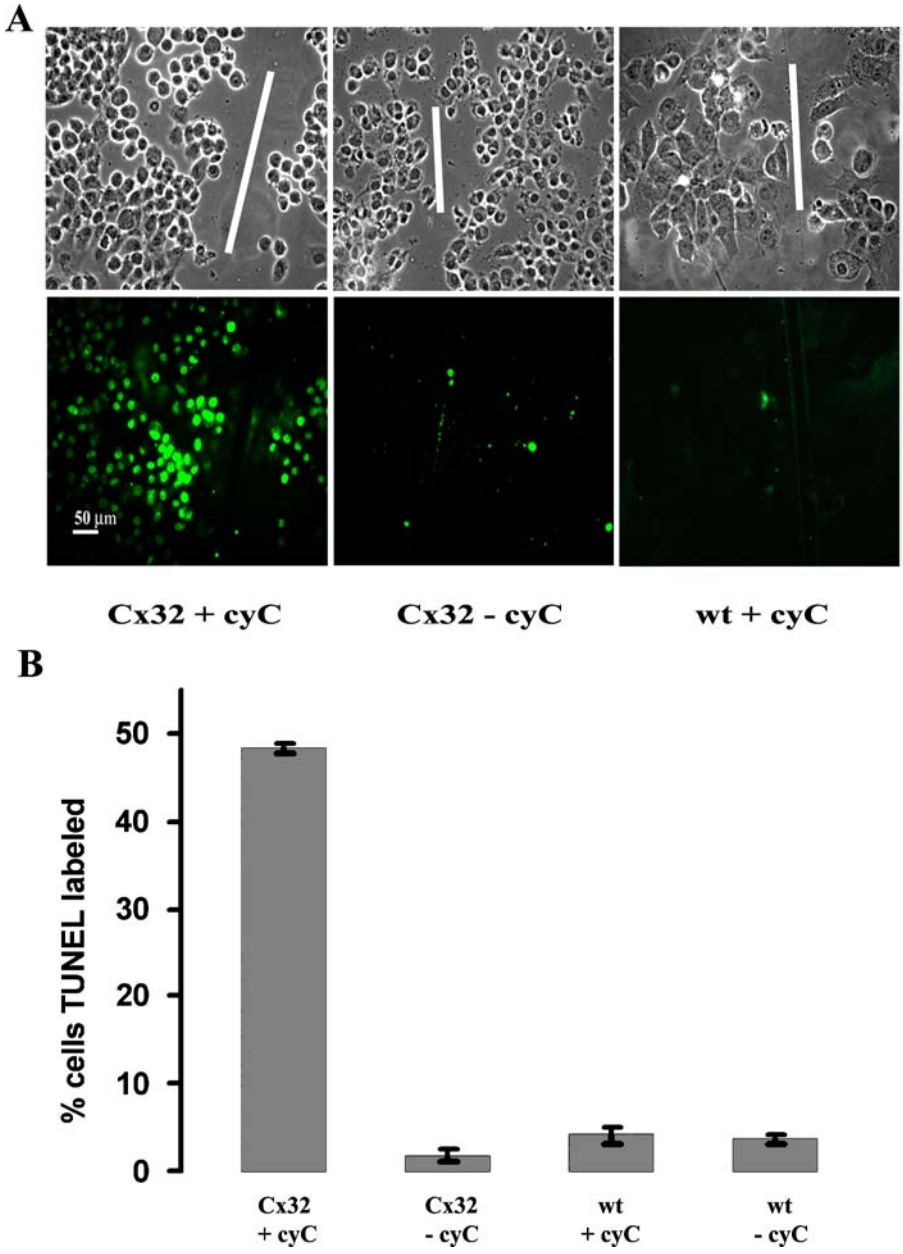


by a white line) were labeled for both dyes, but in the absence of Cx32 neither LY nor the rhodamine-dextran complex had spread to neighboring cells. For the Cx32-transfected BHK cells (shown in the right hand column), the LY accumulated by cells along the scrape was transferred to numerous cells remote from the scrape, whereas the much larger dextran complex was acquired only by those cells injured by the scrape. The histograms of Figure 2B show that approximately the same fraction of BHK wt cells in the monolayer took up Lucifer yellow (14.3% $\pm$ 1.7, mean $\pm$ SEM, $n = 3$) and rhodamine-dextran (12.7% $\pm$ 2.3, mean $\pm$ SEM, $n = 3$). In contrast, the data illustrate that a significantly greater number of BHK cells transfected with Cx32 acquired the Lucifer yellow (32.4% $\pm$ 1.3, mean $\pm$ SEM, $n = 5$) than the larger rhodamine complex (8.3% $\pm$ 1.6, mean $\pm$ SEM, $n = 5$).

Cytochrome C- induced apoptosis: Having shown that GJIC is readily established among cells transfected with Cx32, confluent cells were scrape loaded with cyC to induce cell death and assay for the spread of apoptosis. The presence of cells undergoing apoptosis was investigated by fluorescence microscopy for a variety of indicators: TUNEL staining, the binding of annexin V, and immunostaining of specific cleaved caspases of the apoptosis cascade. In viewing the fluorescent images, it is important to point out a fortuitous consequence of the scrape-loading method. Cells bathed in cyC and injured by the scrape remained in contact with their neighbors, but became loosely adherent to the plastic substrate. As a result, after the extended period of incubation in which the cells lining the cut accumulated cyC— and had the opportunity to transfer death-inducing signals to their neighbors—many were washed away in the multiple rinses used to remove the cyC prior to fluorescence analysis. Since the protein is too large to traverse gap junctions, few cytochrome C-labeled cells immediately adjacent to the scrape are seen in the figures. In order to establish that bystander killing was mediated via gap junctions, several control experiments were carried out in the presence of the gap junctional blockers, octanol and carbenoxolone, and with communication deficient wild-type BHK cells that did not possess functional gap junctions.

After exposure to cyC, there was extensive TUNEL labeling of Cx32-expressing BHK cells near the scrape and throughout the culture dish (Figur 3A, Cx32 + cyC). Cells lacking the gap-junctional protein and exposed to cyC (wt + cyC), and Cx32 expressing BHK cells not treated with cyC (Cx32 − cyC), showed no signs of apoptosis away from the scrape. Clearly, mechanical injury alone did not itself induce a significant number of TUNEL-stained cells, nor did we observe significant apoptotic activity at the site of the scrape with other indicators. A quantitative comparison of the percentage of cells within the population of cells in each experimental dish that exhibited TUNEL staining is shown in Figure 3B for four different experimental conditions. For Cx32 transfected BHK cells, 48.3% $\pm$ 1.14 (mean $\pm$ SEM, $n = 4$) of the cell population ($\sim$120/dish) were TUNEL labeled following exposure to cytochrome C. In contrast, 4.1% $\pm$ 1.1 (mean $\pm$ SEM, $n = 3$) of wild-type BHK cells were stained after scrape loading with cyC, and similar values were obtained for both transfected (1.85% $\pm$ 0.64, mean $\pm$ SEM, $n = 3$) and wt (3.6% $\pm$

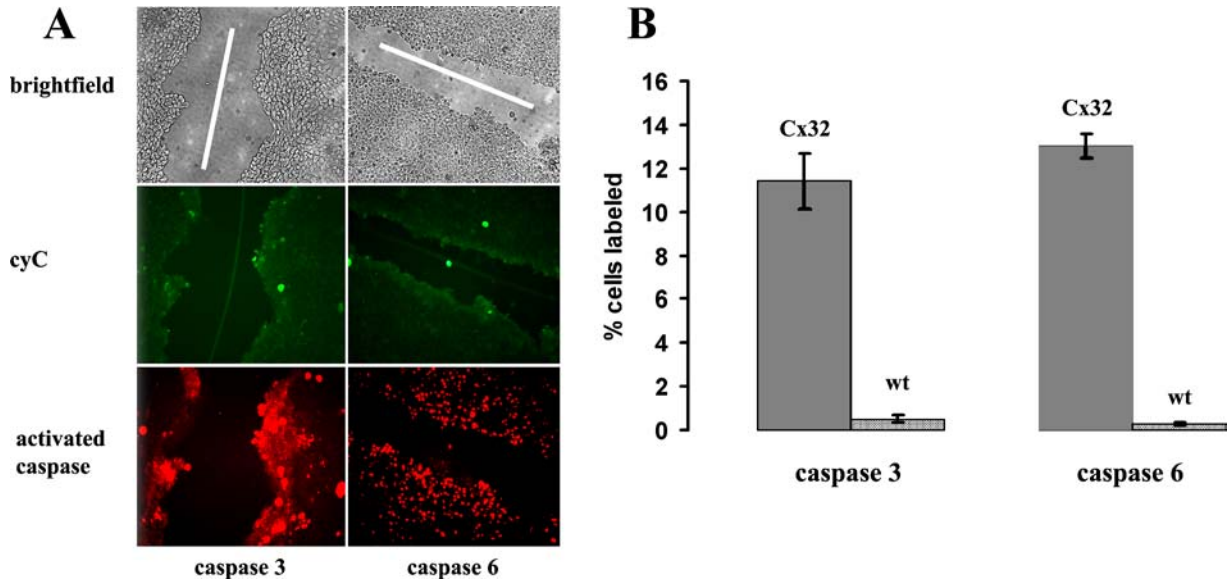
Figure 3. (A) TUNEL labeling of apoptotic BHK cells scrape loaded with cyC. Bright field images (upper row) show the location of the scrape (bar) and the cells after exposure to cytochrome c, multiple rinses, and TUNEL labeling with fluorescein-dUTP nucleotide. Cells transfected with Cx32 (lower left) show extensive spread of the fluorescent tag, whereas few cells undergoing apoptosis are evident in communication-deficient BHK-wt cells, whether exposed or not to cytochrome C. (B). Bar graphs show the fraction of TUNEL labeled cells under various experimental conditions; see text for details.



0.48, mean ± SEM, $n = 3$) cells when scraped but not exposed to cyC. Thus it is apparent that the inclusion of cyC did not produce more extensive cell death in non-communicating cells than would occur simply by cutting the cell monolayer.

The presence of cleaved caspases 3, 6, and 9 was also seen after induction of apoptosis with cytochrome C in Cx32-transfected BHK cells under the conditions described above (Figure 4). Figure 4A shows that relatively few cells immunoreactive to cyC are seen along the scrape (central images), but there is widespread distribution of cells distant from the scrape that were immunostained with antibodies for the activated forms of effector caspases 3 and 6 (Figure 4, bottom row). These results are illustrated quantitatively in the histograms of Figure 4B. For Cx32 transfected cells exposed to cyC, caspase3 expression was seen in 11.4% ± 1.3 (mean ± SEM, $n = 4$) of the cells, whereas only 0.5% ± 0.1 (mean ± SEM,

Figure 4. (A) Immunocytochemistry for the presence of cleaved caspases 3 and 6 after induction of apoptosis in Cx32-transfected BHK cells. Bright field images (upper row) show the orientation of the scrape (white bar), and the cells after exposure to cyC and the subsequent fixation procedures. Activation of caspases 3, and 6 extended to coupled cells distant from the scrape (bottom row). (B) Bar graphs compare the activation of caspases 3 and 6 in wild type (light shading) and Cx32 transfected (dark shading) BHK cells scrape loaded with cytochrome C.



$n = 4$) of wt cells exposed to cyC were labeled. A similar result was seen with activated caspase 6 where 13% ± 0.6 (mean ± SEM, $n = 4$) of the Cx32-expressing cells exposed to cyC were labeled, compared to only 0.3% ± .07 of wt cells treated with cyC.

Another quantitative index of the results obtained with the various apoptosis assays is shown in the bar graphs of Figure 5. This series of experiments, each of which comprised three or four individual runs, provided comparative data for Cx32-transfected BHK cells and wild-type BHK cells after exposure to cytochrome C. Except for the annexin-V results, the connexin-transfected cells exhibited a more than 10-fold increase in the amount of cell death induced by cyC; for the annexin study, there was a >5-fold increase in staining when Cx32 was expressed. It is important to note that the discrepancy between the levels of apoptosis (as indicated by TUNEL staining) and activation of the various caspases results from the transient nature of caspase cleavage, *i.e.*, the sequential series of active proteases and other substrates that lead to the final stage of DNA degradation revealed by TUNEL. Thus, the levels of TUNEL staining and of the activated caspases do not bear direct comparison with each other, since they do not represent the same time point in the apoptotic cascade. Rather they are compared in each instance with results obtained *in the absence of gap junctions* (*i.e.*, in wild type BHK cells), and as such, provide strong evidence that the presence of gap junctions formed by Cx32 results in the intercellular spread of apoptosis to coupled cells removed from the site of the scrape.

A further indication that the spread of apoptosis was mediated by GJIC is illustrated in Figure 6, which shows the effects of pre-treating the Cx32-transfected BHK cells with the gap junction blockers, carbenoxolone and octanol, prior to scrape loading cyC. Note that the extensive spread of apoptosis, indicated by the percentage of untreated cells labeled for the presence of the various indicators of apoptosis, is grossly suppressed by both of these agents. Indeed, the numbers of apoptotic cells seen in cultures exposed to carbenoxolone or octanol were not significantly different from what was observed with wt cells exposed to cyC; *i.e.*, in either case, apoptosis was confined almost exclusively to injured cells that took up the cytochrome C.

Discussion

The spread of cell death from cells undergoing apoptosis to otherwise unaffected cells has come to be known as the 'bystander effect', a term introduced initially to account for the widespread tumor regression seen with a then novel form of gene therapy.[2] In that study, the herpes simplex virus thymidine kinase gene (HSV-TK) was transfected via a viral vector into tumor cells and exposed to the prodrug ganciclovir, which when phosphorylated by the kinase terminated DNA polymerization and caused the death of the replicating cells. However, although relatively few tumor cells acquired HSV-TK, tumor regression extended throughout the mass to bystander cells that

Figure 5. The percentage of cells labeled for the various indicators of apoptosis. A graphic comparison of BHK cells expressing Cx32 with those lacking the connexin (wt).

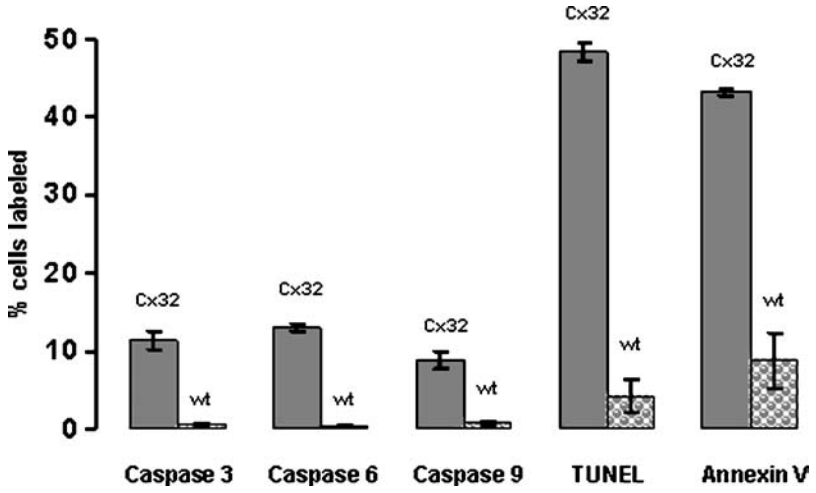
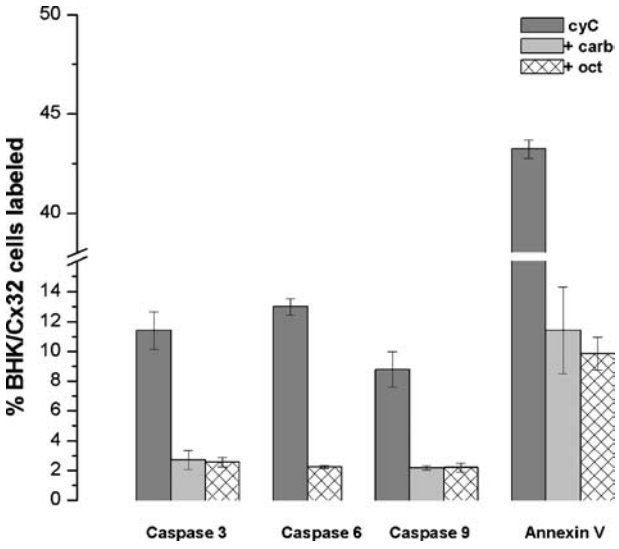


Figure 6. Immunocytochemistry for annexin V and cleaved caspases (3, 6, and 9) show that labeling of cells scrape loaded with cyC (dark gray shading) is greatly reduced in the presence of carbenoxolone (light gray) and octanol (cross hatching).

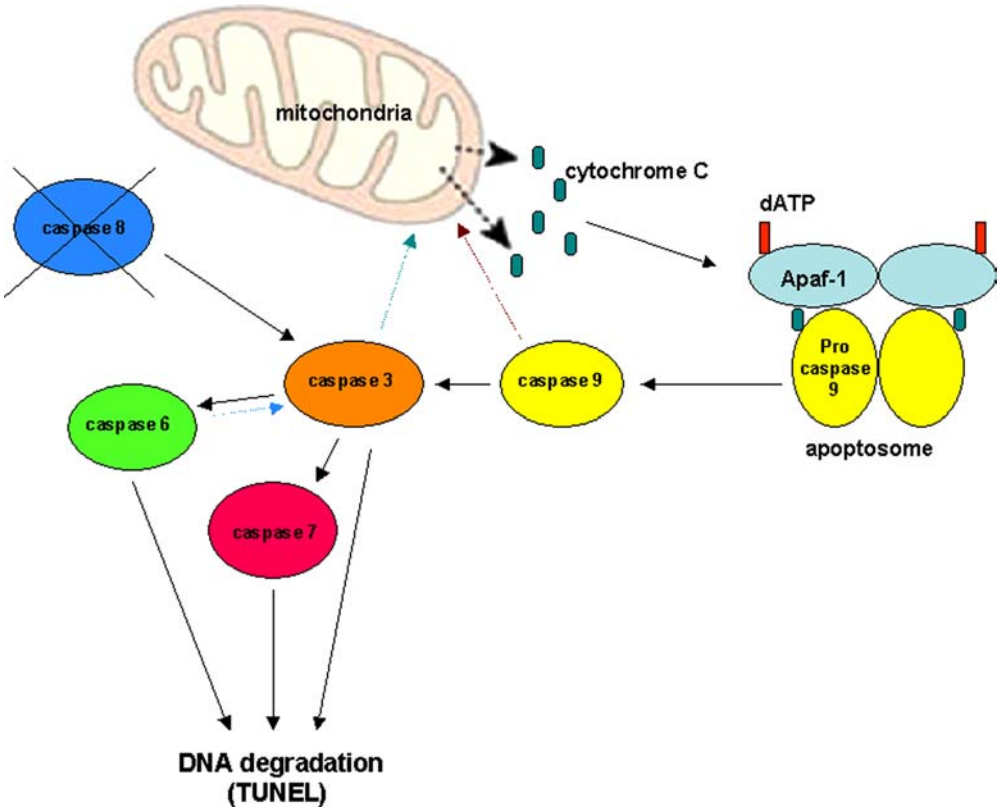


had not incorporated the gene. Subsequent studies have shown that the efficacy of the treatment is due to the intercellular transfer via gap junctions of the toxic metabolites of ganciclovir from the HSV-TK- positive cells to cells that do not express the gene.[3,4,19]

In the present study, a somewhat analogous result was obtained. A culture of largely confluent, normal replicating BHK cells that had been stably transfected with connexin32, were scrape loaded with cytochrome C to allow the protein to enter the injured cells and induce cell death. Nevertheless, neighboring cells remote from the scrape that had not accumulated cyC underwent apoptotic cell death as evidenced by TUNEL staining (Figure 3) and the presence of activated caspases (Figure 4). It is likely that this enzymatic cascade was triggered either by the loss of essential metabolites or the acquisition of a death-inducing product generated during apoptosis. In either event, the transfer had taken place via the gap junctions through which the cyC cells and the adjoining bystander cells were coupled. When these intercellular pathways were absent or blocked by octanol or carbenoxolone (Figure 6), cell death was restricted to cells that had incorporated the cyC at the site of the scrape. It is noteworthy that comparable results were obtained in a recent study[20] in which pairs of *Xenopus* oocytes were electrically coupled through their endogenous connexin (Cx38). Injection of cyC into one of the oocytes caused a progressive loss of membrane potential and subsequent death in both the injected and non-injected cell over a period of 2–3 hrs. Gross changes in cell structure were readily visible as the cells died, but in contrast to the results shown here, the spread of apoptosis was limited to the one oocyte coupled to the injected cell, and the opaque cytoplasm of the oocyte made it difficult to use the various fluorescent assays of apoptosis that proved so revealing in the present study.

The use of cytochrome C to induce cell death provided a convenient means to circumvent the need for various extracellular signaling molecules that activate membrane receptors of the death domain family such as Fas, TRAIL or tumor necrosis factor (TNF). There is good evidence that cytoplasmic cyC is an important component of the apoptotic cascade,[21] and that its release from mitochondria precedes exposure of phosphatidylserine, the loss of plasma membrane integrity, and other indices of apoptosis.[15] The precise mechanism by which the protein is released from mitochondria is not fully understood, and subsequent events leading to apoptosis can be suppressed by Bcl-2

Figure 7. Schematic representation of the pathways involved in apoptosis following the mitochondrial release - or intracellular injection— of cytochrome C. In the presence of dATP (or ATP), Apaf-1, pro-caspase 9, and cytochrome C , an 'apoptosome' is formed which results in the activation of caspase 9. This leads, in turn, to activation of effector caspases 3, 6, and 7, and ultimately to DNA degradation (TUNEL labeling) and cell death. Cleavage of caspase 8, another cysteine-dependent aspartate-directed protease that can induce apoptosis, is not a component of the mitochondria-associated "intrinsic apoptotic pathway" triggered by the release of cytochrome C. For simplicity, other endonucleases and related events known to occur in this cascade, but not examined in this study, have been omitted.



acting at various loci at, or in proximity to, the outer mitochondrial membrane.[22] However, as shown in Figure 7, this machinery is also bypassed when cyC is introduced intracellularly,[23–26] whereupon it forms a complex (the so-called apoptosome) with Apaf-1, procaspase- 9 and dATP. The resultant cleavage and activation of caspase 9[27–29] leads, in turn, to the activation of downstream executioner caspases (*e.g.*, 3 and 6) that cause DNA damage, disruption of cytoskeletal proteins and the stereotypical changes that characterize apoptotic cell death.[30,31]

Interestingly, maximum fluorescent signals associated with activation of the various caspases occurred at different times, approximately in accordance with the sequence of events depicted in Figure 7. A series of preliminary experiments performed at different times after exposure to cyC revealed that a strong fluorescent signal for cleaved caspase 9 was observed less than 15 minutes after induction of apoptosis, but fluorescence was barely detectable after about 1 hr. On the other hand, immunostaining associated with activated caspase 3 was evident after ~30 min, was maximal after about 1 hr, and continued for up to 1.5 hours after exposure to cyC (data not shown). Maximum staining for activated caspase 6 was also observed about one hour after induction of apoptosis. These observations are consistent with data indicating that caspase 9 is the upstream caspase in the protease cascade that leads to apoptosis.[27] Similar results were obtained with the binding of annexin V, which typically occurs as an early event in apoptosis. However, we found no evidence by immunocytochemistry that caspase 8 had been activated, in keeping with earlier observations indicating that cleavage of caspase 8 is not a component of the mitochondria-associated "intrinsic apoptotic pathway" triggered by the release of cytochrome C.[21,30]

Although programmed cell death plays an essential role in normal development and in the regulation of cell proliferation, many of its key features are seen frequently in pathological conditions. The process can be initiated by a variety of stimuli acting through numerous membrane receptors, and a myriad of intracellular pathways that lead to the hallmark biochemical and morphological reactions that characterize apoptosis have been identified.[18,30,32,33] However, the factors that trigger these events in inherited neurodegenerative diseases are poorly understood. In the

human retina, for example, a large number of point mutations in rhodopsin and other rod-specific proteins are associated with retinitis pigmentosa,[34] a broad spectrum of blinding diseases that result in widespread apoptotic death of rod photoreceptors.[35] However, a particularly perplexing problem typical of these disorders is the death of genetically normal cone photoreceptor cells that tend to follow the pattern of death exhibited by their neighboring rods. To account for this phenomenon, it has been postulated[36] that dying rods transmit noxious agents to neighboring cones via the gap junction channels that connect rods and cones in the mammalian retina.[37–39] The results of the present study, and recent reports by Cusato *et al*.[20] and Krutovskikh *et al*.[40] lend credence to this notion.

Conclusion

As witnessed by the dye transfer assay (Figure 2), Lucifer yellow was able to travel intercellularly via gap junctions because of its small size, whereas the larger tetramethylrhodamine dextran complex remained confined within the cells it entered near the scrape. In this connection, it is important to stress that cytochrome C (MW = 12,327; d = 34 Å) as well as the various caspases activated in the course of apoptosis are too large to traverse the pores of gap-junctional channels. Thus, the cell death signals must be conveyed by ions or small molecules that are generated as a result of exposure to cyC. A rise in Ca^{2+}, pH changes, and the accumulation of cAMP, IP_3, ATP and reactive oxygen species are some of the events that can occur in apoptotic cells,[41–45] and are among the potential candidates that could traverse gap junctions to induce bystander cell death. The connexin-expressing BHK cell preparation lends itself to analysis of many of these factors by a variety of biochemical, imaging, and related methods, and we are currently using a number of these approaches to identify the intercellular messenger(s) that mediate the bystander effect.

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