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Research Article

Connexin43 increases the sensitivity of prostate cancer cells to TNFα-induced apoptosis

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Summary

To examine the effects of increased expression of connexin43 (Cx43) upon cell viability and response to cytotoxic agents, we expressed Cx43 in LNCaP and PC3 prostate cancer cells by infection with a recombinant adenovirus (Ad-Cx43). Infection with Ad-Cx43 led to the formation of Cx43-containing gap junction plaques at appositional membranes and increased Lucifer Yellow transfer in LNCaP cells, but not in PC3 cells. The increased intercellular communication was blocked by co-infection with an adenovirus containing a dominant-negative Cx43 (Ad-Cx43DN). Infection of LNCaP (but not PC3) cells with Ad-Cx43 greatly increased their sensitivity to killing by tumor necrosis factor α (TNFα), anti-Fas antibodies, and TRAIL as quantified using an MTS assay. The TNFα-induced cell death was dependent on cell density, and it was associated with increased annexin V staining, an increased proportion of sub-G1 cells, and activation of caspase 8. The TNFα-induced effects on Ad-Cx43-infected LNCaP cells were blocked by co-infection with Ad-Cx43DN or by pre-incubation with neutralizing antibodies directed against TNFα receptor 1. These results demonstrate that TNFα induces apoptosis in LNCaP cells by signaling through TNFα receptor 1 and that expression of functional Cx43 gap junction channels increases their sensitivity to TNFα.

Key words: Gap junction, Intercellular communication, Connexin, Apoptosis, TNFα

Introduction

Many cancer cells (from a large variety of species and tissues) show alterations or absence of intercellular communication (Loewenstein and Kanno, 1966). Thus, it has long been hypothesized that reduced gap junction-dependent coupling among transformed cells or between them and their normal neighbors contributes to the cancer phenotype, including loss of growth control (Vinken et al., 2006). Gap junctions are membrane specializations containing clusters of intercellular channels. These channels are oligomeric assemblies of members of a family of related proteins called connexins (Cx). Six connexin monomers assemble to form a hemi-gap junction channel or connexon, which, in turn, forms a complete gap junction channel by docking with a connexon from an adjacent cell. Gap junction channels allow intercellular passage of ions and molecules of up to 1000 Da, including second messengers.

Gap junction-mediated intercellular communication may also influence the response of cancer cells to treatments by facilitating the passage of toxic drug metabolites or death signals (that induce or augment apoptosis) between coupled cells (Andrade-Rozental et al., 2000; Krysko et al., 2005; Trosko and Ruch, 2002; Udawatte and Ripps, 2005). Connexin expression and intercellular communication have been linked to the 'bystander effect' seen in several types of cytotoxicity. The most extensively studied phenomenon involves the Herpes simplex virus-thymidine kinase (HSV-TK)/ganciclovir approach to tumor gene therapy (Colombo et al., 1995; Fick et al., 1995; Mesnil et al., 1996) where it has been suggested that a ganciclovir metabolite generated in cells expressing thymidine kinase might traverse gap junction channels to kill neighboring, non-kinase-expressing cells. Some studies have suggested that gap junction channels might facilitate the passage of 'death signals' from lethally irradiated cells to their neighbors (Azzam et al., 2004; Zhou et al., 2002). Similarly, gap junctions may augment the killing of cells by cisplatin (Jensen and Glazer, 2004; Tanaka and Grossman, 2001). In the nervous system, connexins have been proposed to facilitate both cell death and survival (Blanc et al., 1998; Lin et al., 1998; Ozog et al., 2002).

Many cancer cells are characterized by dysfunction of apoptosis as well as intercellular communication (Mesnil et al., 2005; Trosko et al., 2004). While normal prostate cancer epithelial cells form gap junctions, gap junctional communication is either absent or reduced in a variety of prostate cancer cell lines (Mehta et al., 1996).

We chose to study gap junctions and their influence upon the response to cancer therapies in two well characterized cell lines that may represent different stages in the progression of prostate cancer. PC3 cells are androgen-independent, deficient in cell adhesion, and invasive. LNCaP cells are more differentiated, hormone-sensitive, and less metastatic. Both cell lines are deficient in gap junctions, but expression of connexins is sufficient to restore intercellular communication only in LNCaP cells (Govindarajan et al., 2002; Mehta et al., 1999).

The data presented demonstrate that connexin expression can greatly increase the susceptibility of some prostate cancer cells (LNCaP) to agents (TNFα, TRAIL and anti-Fas
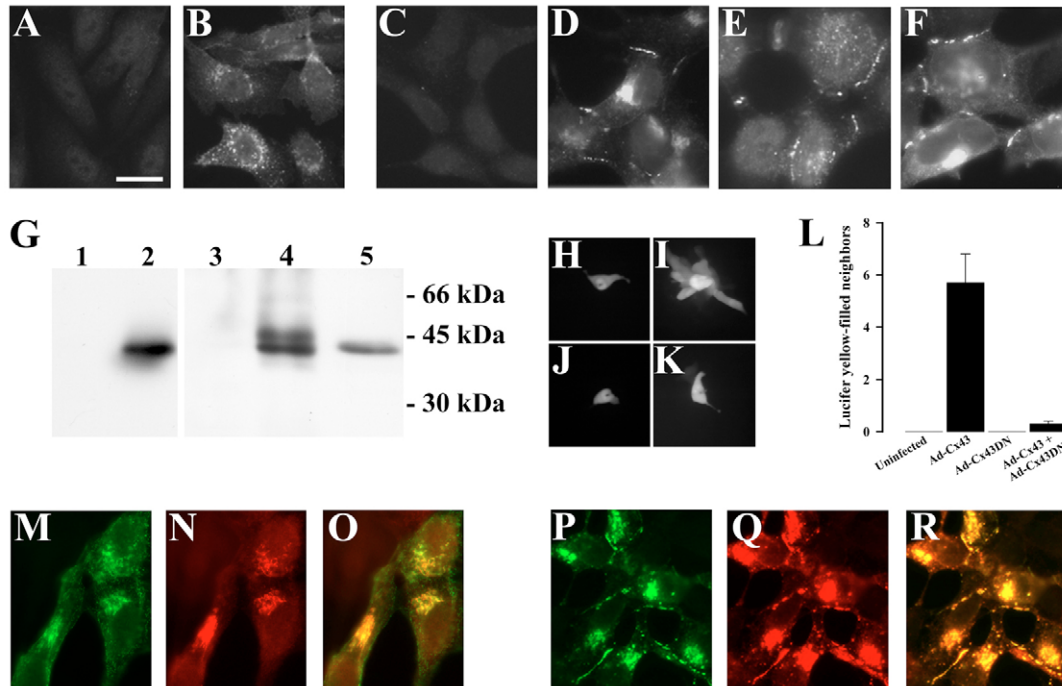
Fig. 1. Cx43 induces the formation of functional gap junction channels in LNCaP but not PC3 cells. (A,B) Photomicrographs show the distribution of Cx43 immunoreactivity in PC3 cells that were uninfected (A) or infected with Ad-Cx43 (B). (C-F) Photomicrographs show the distribution of connexin immunoreactivity in LNCaP cells that were uninfected (C) or infected with Ad-Cx43 (D), Ad-Cx37 (E), or Ad-Cx40 (F). (G) Immunoblot detection of Cx43 in cell lysates from PC3 cells that were uninfected (lane 1) or infected with Ad-Cx43 (lane 2) and in lysates of uninfected LNCaP cells (lane 3) or LNCaP cells infected with Ad-Cx43 (lane 4) or Ad-Cx43DN (lane 5). The migration positions of the molecular mass markers are indicated on the right. (H-K) Representative fluorescence images of Lucifer Yellow transfer after microinjection of the tracer into a single cell within a cluster of uninfected LNCaP cells (H) or LNCaP cells infected with Ad-Cx43 (I), Ad-Cx43DN (J), or both Ad-Cx43 and Ad-Cx43DN (K). (L) Histogram showing the extent of intercellular transfer of Lucifer Yellow in uninfected LNCaP cells and LNCaP cells infected with Ad-Cx43, Ad-Cx43DN, or both adenoviruses. Values represent the number of recipient cells containing Lucifer Yellow (mean ± s.e.m., n=8). (M-O) Photomicrographs show the localization of Cx43DN (M) and Golgi 58K protein (N) in LNCaP cells infected with Ad-Cx43DN after double-labeling immunofluorescence using anti-Cx43 and anti-Golgi 58K antibodies. The overlap of the two signals appears yellow in the merged image (O). (P-R) Photomicrographs show the localization of Cx43 and Cx43DN in LNCaP cells co-infected with Ad-Cx43 and Ad-Cx43DN after double-labeling immunofluorescence using anti-Cx43IL (P) and anti-HA (Q) antibodies. The overlap of anti-Cx43IL and anti-HA immunoreactivities appears yellow (R). Bar, 20 μm for A-D and P-R, 8 μm for E, 12 μm for F, 66 μm for H-K and 17 μm for M-O.

antibodies) that induce apoptosis and thus implicate, for the first time, the participation of connexin-dependent functions in the extrinsic pathway of apoptosis.

Results

Manipulation of gap junctions in prostate cancer cells

PC3 and LNCaP cells were characterized for their Cx43 expression. Consistent with previous reports (Govindarajan et al., 2002; Mehta et al., 1999), Cx43 immunoreactivity was not detected in PC3 or LNCaP cells by immunofluorescence or immunoblotting (Fig. 1A,C,G, lanes 1, 3).

To examine the consequences of Cx43 expression in prostate cancer cells, we infected PC3 and LNCaP cells with Ad-Cx43. After infection with Ad-Cx43, immunoreactive Cx43 was only detected within the cytoplasm of PC3 cells (Fig. 1B). By contrast, LNCaP cells infected with Ad-Cx43 showed staining at appositional membranes and in the cytoplasm (Fig. 1D). A similar distribution of connexins was observed when LNCaP cells were infected with Ad-Cx37 or Ad-Cx40 (Fig. 1E,F). Immunoreactive Cx43 was detected as a single band in immunoblots of homogenates prepared from Ad-Cx43-infected PC3 cells (Fig.

1G, lane 2), but as several bands of 40-46 kDa in homogenates from Ad-Cx43-infected LNCaP cells (Fig. 1G, lane 4).

Intercellular communication of Ad-Cx43-infected PC3 and LNCaP cells was assessed by microinjection of the fluorescent tracer, Lucifer Yellow. Neither non-infected nor Ad-Cx43-infected PC3 cells showed transfer of Lucifer Yellow to neighboring cells (data not shown). Uninfected LNCaP cells did not transfer Lucifer Yellow to neighboring cells (Fig. 1H,L); however, infection of these cells with Ad-Cx43 induced intercellular transfer of Lucifer Yellow, with the dye passing to about six neighboring cells on average (Fig. 1I,L). No transfer of Lucifer Yellow was observed in LNCaP cells infected with Ad-Cx37 or Ad-Cx40 (n=10 for each adenovirus); however, extensive transfer of neurobiotin (co-injected with Lucifer Yellow) was always observed in LNCaP cells expressing either Cx37 or Cx40. Many neurobiotin containing cells (20-60) were observed after injection of the tracer into these cells. These results are consistent with our previous observation that infection of HeLa cells with either Ad-Cx37 or Ad-Cx40 allowed extensive transfer of neurobiotin, but little transfer of Lucifer Yellow (Wang et al., 2005).

Because a Cx43 mutant with a deletion of amino acids from the cytoplasmic loop (Cx43DN) has been effective as a 'dominant negative' inhibitor of gap junction function in other cell types (Krutovskikh et al., 1998; Oyamada et al., 2002; Wang et al., 2005), we tested the effects of Ad-Cx43DN infection on LNCaP cells and Cx43-expressing LNCaP cells. In LNCaP cells infected with Ad-Cx43DN, a single immunoreactive band was detected by immunoblotting (Fig. 1G, lane 5), and immunoreactive Cx43 localized in a perinuclear region (Fig. 1M). The distribution of Cx43DN extensively overlapped with the Golgi 58K protein (Fig. 1M-O), but it did not show much overlap with the distribution of PDI, an ER-resident protein (data not shown). Infection with Ad-Cx43DN did not induce intercellular transfer of Lucifer Yellow (Fig. 1J,L). In cells infected with both Ad-Cx43 and Ad-Cx43DN, immunoreactive Cx43 and Cx43DN showed similar distributions at appositional membranes and within the cytoplasm (Fig. 1P,Q) with a high degree of overlap (Fig. 1R). Co-infection with Ad-Cx43 and Ad-Cx43DN resulted in a significant reduction in Lucifer Yellow transfer (nearly to zero) as compared to LNCaP cells infected with Ad-Cx43 alone (Fig. 1K,L).

Cx43 increases susceptibility of LNCaP cells to TNFα-induced cell death

We examined the effects of Cx43 expression upon the sensitivity of the prostate cancer cell lines to killing by various agents that induce apoptosis. Cells were observed for morphological evidence of cell death, and cytotoxity was quantified using an MTS assay. PC3 cells (uninfected or Ad-Cx43 infected) were highly resistant to killing by TNFα at all doses tested ($\leq$100 ng/ml) (Fig. 2A). Etoposide, doxorubicin and staurosporine induced some death of PC3 cells, but no differences were observed between the dose-response killing curves obtained from uninfected or Ad-Cx43-infected PC3 cells (data not shown).

Uninfected and Ad-Cx43-infected LNCaP cells did not differ in their response to the cytotoxic effects of etoposide, doxorubicin and staurosporine (data not shown). By contrast, whereas TNFα treatment had little observable effect upon uninfected LNCaP cells or LNCaP cells infected with control virus, it induced extensive cell death (rounding and detachment) in Ad-Cx43-infected LNCaP cells. Treatment with TNFα ($\geq$0.1 ng/ml), significantly reduced cell viability in Ad-Cx43-infected LNCaP cells as compared to uninfected cells or cells infected with Ad-Control (Fig. 2B).

To test the importance of connexin function in the increased sensitivity of Ad-Cx43-infected LNCaP cells to TNFα, LNCaP cells were infected with both Ad-Cx43 and Ad-Cx43DN. Co-expression of the dominant negative Cx43 mutant completely antagonized the effects of Ad-Cx43. The viability of cells infected with both viruses was significantly greater than that of Ad-Cx43-infected cells at TNFα concentrations of $\geq$0.1 ng/ml, and it was restored to levels indistinguishable from those of uninfected cells at all doses of TNFα tested (Fig. 2C).

To distinguish between the involvement of connexin hemi-channels or gap junction channels in the Cx43-dependent sensitization of LNCaP cells to TNFα, LNCaP cells, plated at different densities, were infected with Ad-Cx43, and cell viability was assessed after treatment with different concentrations of TNFα. Cx43-expressing LNCaP cells were
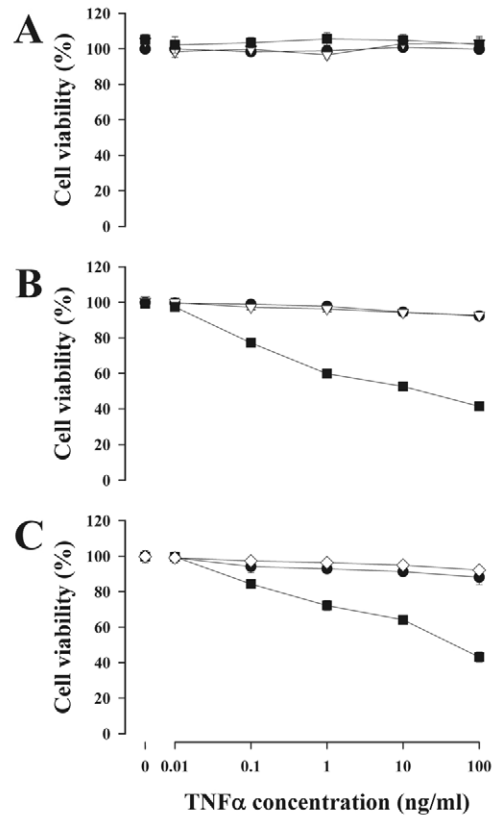

Fig. 2. Cx43 increases the susceptibility of LNCaP cells to TNFα-induced cell death. (A) PC3 cells were left uninfected ($\triangledown$) or infected with Ad-Control ($\bullet$) or Ad-Cx43 ($\blacksquare$) for 12 hours and then treated with 0.01-100 ng/ml TNFα for 48 hours. (B) LNCaP cells were left uninfected ($\triangledown$) or infected with Ad-Control ($\bullet$) or Ad-Cx43 ($\blacksquare$) for 12 hours and then treated with 0.01-100 ng/ml TNFα for 48 hours. Cell viability of untreated and Ad-Control infected cells showed a slight, but insignificant decline with increasing doses of TNFα. These curves did not differ significantly from each other. Ad-Cx43-infected cells showed significantly less viability at 0.1, 1, 10, and 100 ng/ml TNFα ($P<0.0001$). (C) LNCaP cells were infected with Ad-Control ($\bullet$), Ad-Cx43 ($\blacksquare$), or both Ad-Cx43 and Ad-Cx43DN ($\diamond$) for 12 hours and then treated with 0.01-100 ng/ml TNFα for 48 hours. The viability of Ad-Control- or Ad-Cx43 + Ad-Cx43DN-infected cells did not differ from each other. The viability of cells infected with Ad-Cx43 was significantly lower than that of cells infected with both viruses at 0.1-100 ng/ml TNFα ($P=0.0264$ for 0.1 ng/ml; $P<0.0001$ for 1.0, 10, and 100 ng/ml). The results are presented as mean ± s.e.m. although in many cases the error bars are so small that they are obscured by the symbols.

much more sensitive to TNFα when grown at higher cell density than when grown sparsely (Fig. 3). At the highest cell density tested, all concentrations of TNFα (0.01-10 ng/ml) significantly reduced cell viability compared with untreated Ad-Cx43-infected cells. At the second highest cell density, only 1 and 10 ng/ml TNFα induced significant reductions in cell viability. At the lower cell densities, no significant effects on cell viability were observed. These results suggest that TNFα-induced cell death depended on cell contact.

To examine the effects of Cx43 expression upon the sensitivity of the LNCaP prostate cancer cells to killing by other members of the TNF receptor superfamily, cells were
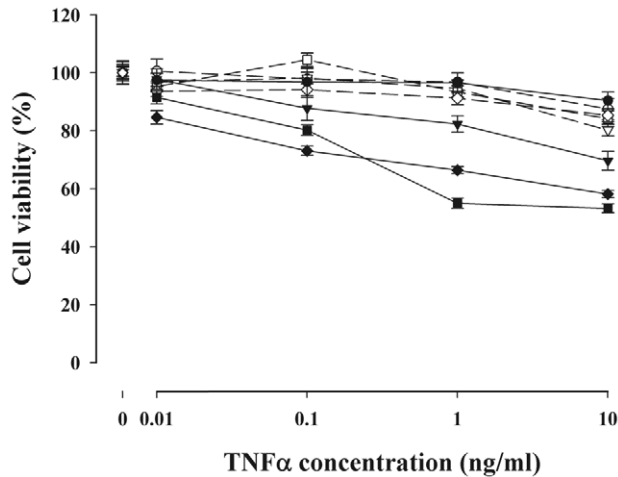
Fig. 3. TNFα-induced cell death in Cx43-expressing LNCaP cells is greater at higher cell densities. LNCaP cells were plated at different densities, 1250 (○, ●), 2500 (△, ▲), 5000 (□, ■) or 10,000 (◇, ◆) initial cells/well. After 12 hours, cells were left uninfected (open symbols) or infected with Ad-Cx43 (filled symbols), incubated for an additional 12 hours and then treated with different concentrations of TNFα for 48 hours, and cell viability was measured using the MTS assay. At the highest density (10000 cells/well), viability of Ad-Cx43-infected LNCaP cells was significantly reduced by all concentrations of TNFα (P=0.0015 for 0.01 ng/ml, P<0.0001 for concentrations ≥0.1 ng/ml) as compared to untreated cells. When the initial cell number was decreased to 5000 cells/well, only TNFα concentrations ≥1 ng/ml induced a significant decrease in cell viability as compared to untreated cells (P<0.0001). At the highest concentrations of TNFα, slight decreases in cell viability were observed for cells plated at 1250 or 2500 cells per well, but these changes were not statistically significant. In uninfected LNCaP cells, TNFα only reduced cell viability significantly when used at 10 ng/ml in cells cultured at the highest initial density (viability=85.3±2.2%, P=0.0052).
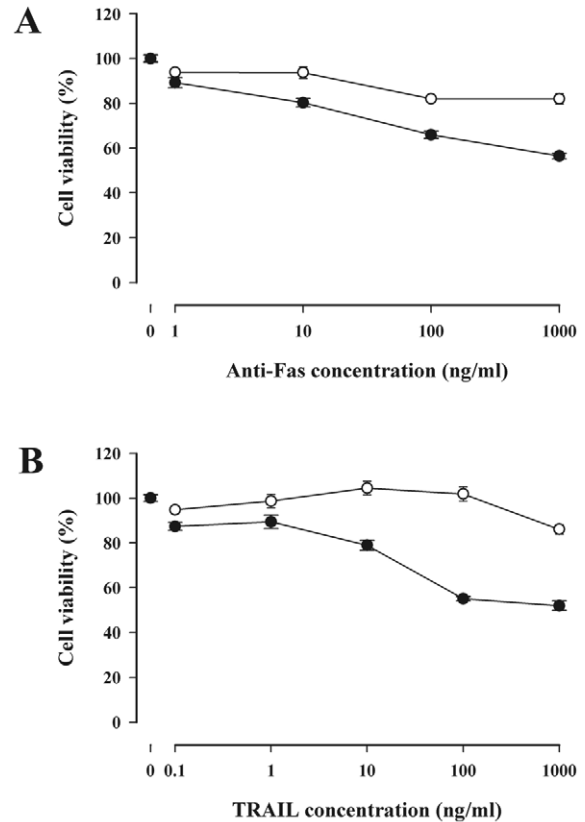

Fig. 4. Cx43 increases the susceptibility of LNCaP cells to cell death induced by anti-Fas antibodies or TRAIL. (A,B) LNCaP cells were left uninfected (○) or they were infected with Ad-Cx43 (●) for 12 hours and then treated with 1-1000 ng/ml anti-Fas antibodies (A) or 0.1-1000 ng/ml TRAIL (B) for 48 hours. Cell viability after treatment with anti-Fas antibodies (≥10 ng/ml) was significantly less in Ad-Cx43 infected LNCaP cells as compared to untreated, infected cells (P<0.0001). Moreover, the viability of Ad-Cx43-infected LNCaP cells treated with ≥10 ng/ml anti-Fas antibodies was significantly reduced as compared to non-infected cells treated with the same doses of antibodies (P<0.0001). High concentrations of anti-Fas antibodies (≥100 ng/ml) moderately, but significantly reduced the viability of uninfected LNCaP cells (to 82.0±1.7% for 100 ng/ml and 81.9±2.3% for 1000 ng/ml, respectively; P<0.0001 as compared to untreated cells). Similarly, in Ad-Cx43-infected LNCaP cells, TRAIL concentrations as low as 10 ng/ml induced a significant decrease in cell viability (P<0.0001), but only 1000 ng/ml TRAIL induced a significant decrease in cell viability of uninfected LNCaP cells (viability=86.0±2.2%; P=0.017 as compared to untreated cells).

treated with anti-Fas antibodies or TRAIL. Uninfected LNCaP cells were not affected by low concentrations of anti-Fas antibodies, but their viability was moderately reduced by treatment with antibody concentrations of ≥100 ng/ml (Fig. 4A). By contrast, the viability of Ad-Cx43-infected LNCaP cells treated with ≥10 ng/ml anti-Fas antibodies was significantly reduced as compared to untreated, infected cells or non-infected cells treated with the same doses (Fig. 4A). Similarly, whereas only 1000 ng/ml TRAIL induced a significant decrease in cell viability in non-infected LNCaP cells, concentrations of TRAIL as low as 10 ng/ml induced a significant decrease in cell viability of Ad-Cx43-infected LNCaP cells (Fig. 4B).

TNFα signals can be transduced by TNFR1 and TNFR2, but only TNFR1 contains a death domain and can directly transmit a death signal; TNFR2 can exert modulatory or synergistic effects on TNFR1 (Baker and Reddy, 1998; Guseva et al., 2004). Therefore, we determined levels of TNF receptors (TNFR) in the prostate cancer cell lines by immunoblotting. TNFR1 and TNFR2 were both detected in PC3 cells (not shown) and in LNCaP cells (Fig. 5A). The levels of TNFR1 or TNFR2 were not affected by infection with Ad-Cx43, treatment with TNFα, or both Ad-Cx43 infection and TNFα treatment. The levels of β-actin, used as

a loading control, remained unchanged under all conditions tested (Fig. 5A).

To test whether the effects of TNFα on Cx43-expressing LNCaP cells were transduced through TNFR1 or TNFR2, we examined the effects of neutralizing antibodies specific for TNFR1 or TNFR2 on LNCaP cell viability. Cx43-expressing LNCaP cells showed significantly greater viability when pretreated with 1 or 10 μg/ml TNFR1 antibody, or 10 μg/ml TNFR1 and 10 μg/ml TNFR2 antibodies, but they were not affected by treatment with TNFR2 antibodies alone (Fig. 5B). These data suggest that the TNFα-induced cell death was mediated through TNFR1.
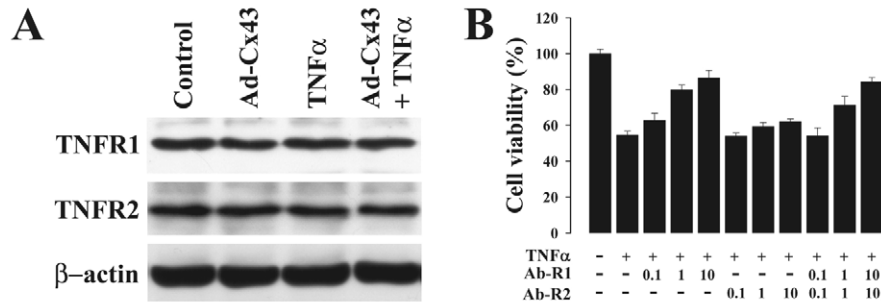
Fig. 5. TNFα induces the death of Cx43-expressing LNCaP cells through TNFR1. (A) Immunoblots showing levels of TNFR1, TNFR2 and β-actin in uninfected LNCaP cells (Control), LNCaP cells 12 hours after infection with Ad-Cx43 (Ad-Cx43), uninfected LNCaP cells after 12 hours of treatment with 10 ng/ml TNFα (TNFα), and LNCaP cells 12 hours after Ad-Cx43 infection and TNFα treatment (Ad-Cx43 + TNFα). (B) Bar graph showing viability of Cx43-expressing LNCaP cells in the presence or absence of neutralizing anti-TNFR1 (Ab-R1) or –TNFR2 (Ab-R2) antibodies (concentrations indicated in μg/ml). Cell viability determined by MTS assay is expressed as a percentage of control values. Statistical analysis of the raw absorbance data showed that treatment with 1 or 10 μg/ml of anti-TNFR1 antibodies or the combination of 10 μg/ml anti-TNFR1 and 10 μg/ml anti-TNFR2 antibodies significantly antagonized the effects of TNFα ($P<0.0001$ when compared to the viability of cells treated with TNFα alone).

To study the specificity of Cx43 effects on the TNFα-induced cell killing, we infected LNCaP cells with recombinant adenoviruses containing sequences encoding two other connexins, Ad-Cx37 and Ad-Cx40, and examined cell viability. TNFα treatment did not produce a significant decrease in cell viability in Ad-Cx37- or Ad-Cx40-infected LNCaP cells as compared to Ad-Control-infected or uninfected cells (data not shown).

TNFα-induced death of Ad-Cx43-infected LNCaP cells occurs through apoptosis

Annexin V and propidium iodide (PI) labeling were quantified by flow cytometry in LNCaP cells that had been infected with adenoviruses and subsequently (12 hours later) treated with TNFα (for a further 12 hours). Spontaneous apoptosis (annexin V labeling) was low in LNCaP cells cultured under control conditions (Fig. 6, top left panel). Infection with either Ad-Cx43 or Ad-Cx43DN did not affect spontaneous apoptosis (Fig. 6, center and lower left panels). Treatment of uninfected cells with TNFα led to a modest increase in the fraction of annexin V- (early apoptosis) and annexin V/PI- (late apoptosis) positive cells (Fig. 6, top right panel). However, the fraction of apoptotic cells was greatest in Ad-Cx43-infected cells treated with TNFα (Fig. 6, middle right panel). Moreover, co-infection with Ad-Cx43 and Ad-Cx43DN largely abolished the fractions of annexin V- or annexin V/PI-positive cells detected following TNFα treatment (Fig. 6, lower right panel). In three independent experiments, the total percentage of annexin V-positive cells ranged between 5.0-9.0 for uninfected TNFα-treated LNCaP cells, 20.4-23.0% for Ad-Cx43-infected cells treated with TNFα, and 3.5-6.0% for cells infected with both Ad-Cx43 and Ad-Cx43DN and treated with TNFα. Annexin V labeling was consistently low in untreated cells that were uninfected or infected with Ad-Cx43 or Ad-Cx43DN (1.8-2.4%, 2.3-3.7% and 1.5-3.4%, respectively).

Cell cycle kinetics were studied by flow cytometry of permeabilized LNCaP cells stained with PI. An apoptotic fraction of cells (containing sub-diploid amounts of DNA) was detected as a 'sub-G1' peak (Fig. 7). In three independent experiments, the sub-G1 fraction was small in untreated/uninfected LNCaP cells (1.2-1.6%) or cells infected with Ad-Cx43 (2.4-3.2%) or Ad-Cx43DN (1.4-1.7%). However, a large sub-G1 fraction (43.6-48.0%) was detected after TNFα treatment of Ad-Cx43-infected LNCaP cells. Co-infection of LNCaP cells with Ad-Cx43 and Ad-Cx43DN and subsequent treatment with TNFα yielded a much lower sub-G1 fraction (15.8-19.2%) that was only a little higher than that in cells treated with TNFα alone (9.7-13.0%).

One of the initial events in the extrinsic pathway of apoptosis is the activation of caspase 8. To determine whether the TNFα-induced apoptosis of LNCaP cells involved caspase 8 activation, we assayed caspase 8 activity at various times after TNFα treatment of Ad-Cx43-infected and Ad-Control-infected LNCaP cells. There was a modest, but significant increase in caspase 8 activity 30 minutes after Ad-Control-infected LNCaP cells were treated with TNFα (Fig. 8A). In Cx43-infected cells, TNFα treatment induced a much greater and more sustained increase in caspase 8 activity. Caspase 8 activity was significantly greater than baseline levels or levels in Ad-Control-infected cells at 30 minutes, 1 hour and 3 hours (Fig. 8A).

To determine the TNFR subtypes involved in the activation of caspase 8, we examined the effects of TNFR neutralizing antibodies upon caspase 8 activation following TNFα treatment of LNCaP cells (Fig. 8B). Only TNFR1 antibodies significantly antagonized the small increase of caspase 8 activity produced in Ad-Control-infected LNCaP cells. In the Cx43-expressing cells, TNFR1 antibodies (alone or together with TNFR2 antibodies) dramatically reduced caspase 8 activity to levels indistinguishable from control values. In addition, TNFR2 antibodies produced a modest, but significant reduction in caspase 8 activity in the Cx43-expressing cells as compared to treatment with TNFα alone. These data suggest that caspase 8 activation was predominantly mediated through TNFR1, but was also affected by activation of TNFR2 receptors.

Discussion

The current study is the first to show that expression of the gap junction protein Cx43, which produced no toxicity by itself
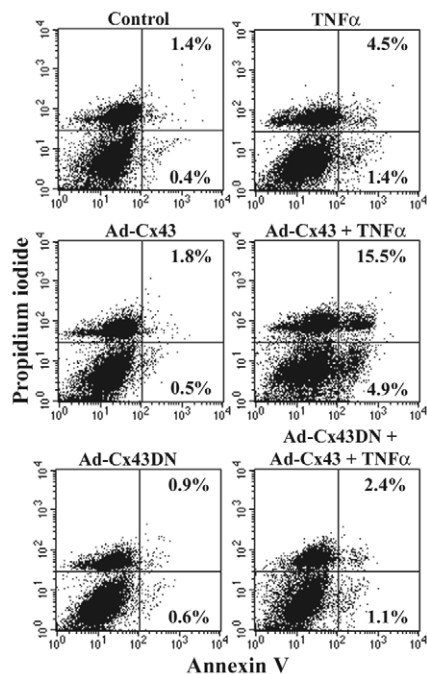
Fig. 6. TNFα induces apoptotic cell death in Cx43-expressing LNCaP cells. Graphs show the distribution of propidium iodide and/or annexin V staining in uninfected or infected LNCaP cells under control conditions or after treatment with 10 ng/ml TNFα for 12 hours. The percentage of cells positive for only annexin V is indicated in the lower right quadrants and that of cells positive for both annexin V and PI is indicated in the top right quadrants.

Fig. 7. TNFα treatment leads to an increase in the percentage of cells in the sub-G1 fraction. Graphs show the distribution of propidium iodide staining intensities in uninfected or infected LNCaP cells under control conditions or after treatment with 10 ng/ml TNFα for 48 hours. The percentage of cells containing a hypodiploid amount of DNA (sub-G1) is indicated.

greatly enhanced the killing of some prostate cancer cells by TNFα. Our data regarding annexin V/PI staining, cell cycle analysis, and caspase 8 activity confirmed that this cell death was due to apoptosis.

Different cancer cells (including those derived from the prostate) differ in their sensitivities (or resistances) to TNFα. Our data extend the results previously obtained with the PC3 and LNCaP cell lines, and they implicate intercellular communication in determining TNFα sensitivity. Prior studies have shown that PC3 cells are quite resistant to TNFα-induced cytotoxicity; they are unaffected by concentrations as high as 200 ng/ml (Chopra et al., 2004; Chung et al., 1998; Nakajima et al., 1996; Yu et al., 2000), but killing may be induced by 1 μg/ml TNFα (Rokhlin et al., 1997). Consistently, we found no effects of TNFα (at concentrations as high as 100 ng/ml) upon PC3 cells. LNCaP cells are also relatively resistant to TNFα; typically, concentrations of at least 10-20 ng/ml have been required to see any evidence of apoptosis (Condorelli et al., 1999; Kulik et al., 2001; Lee et al., 2003). We found little TNFα-induced cytotoxicity in LNCaP cells (uninfected or infected with control adenovirus). However, introduction of Cx43 greatly accentuated the sensitivity of LNCaP to killing by TNFα. Ad-Cx43-infected cells were killed by very low doses (with significantly decreased cell viability seen even at 0.1 ng/ml), and the cytotoxicity increased in a concentration-dependent manner. The apparent discrepancy in the level of apoptosis in TNFα-treated Cx43-expressing cells as determined by annexin V staining compared to that determined
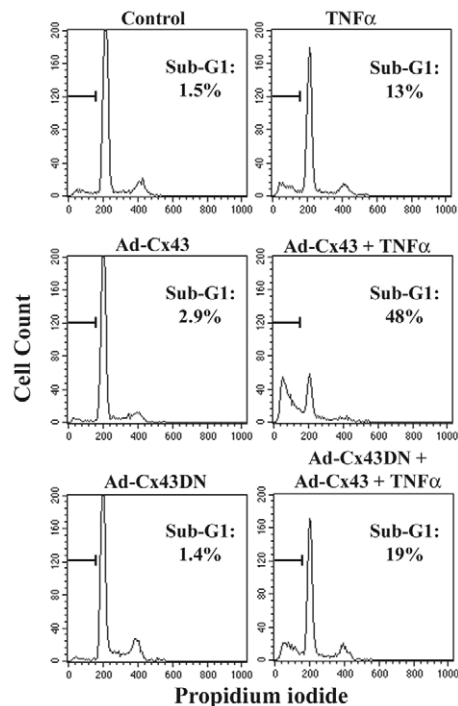
by cell cycle analysis results from the difference in duration of the TNFα treatment. Annexin V staining was determined after 12 hours of treatment to allow detection of the sub-population of cells that had exposed phosphatidyl serine on the external surface of the plasma membrane, but had not yet lost membrane integrity, which represents an early event in apoptosis; thus, in Fig. 6, 4.9% of cells were positive for annexin V alone out of a total of 20.4% annexin V-positive cells. By contrast, the sub-G1 population (43.6-48.0%) was determined after 48 hours of TNFα treatment. This result is consistent with the cell viability of 52.6±1.8% shown in Fig. 2B for Ad-Cx43-infected LNCaP cells treated with 10 ng/ml of TNFα.

In our studies, viability of the LNCaP cells was not affected by infection with Ad-Cx43, Ad-Cx43DN, or control adenovirus. Introduction of connexins and manipulation of intercellular communication have had different effects on cell viability and growth in different kinds of normal and transformed cells (Eghbali et al., 1991; Mehta et al., 1991; Mesnil et al., 1995; Naus et al., 1992). We previously found no effect of Ad-Cx43 on the viability of endothelial (Seul et al., 2004) or HeLa (Wang et al., 2005) cells. Other studies have reported that retroviral introduction of Cx43 or Cx32 (Mehta et al., 1999) and adenoviral introduction of Cx26 (Tanaka and Grossman, 2004) inhibited the growth of LNCaP cells. Our experiments were performed over a shorter time period (48 h or less) than those of Mehta et al., who found that either Cx32 or Cx43 expression inhibited colony formation by LNCaP cells
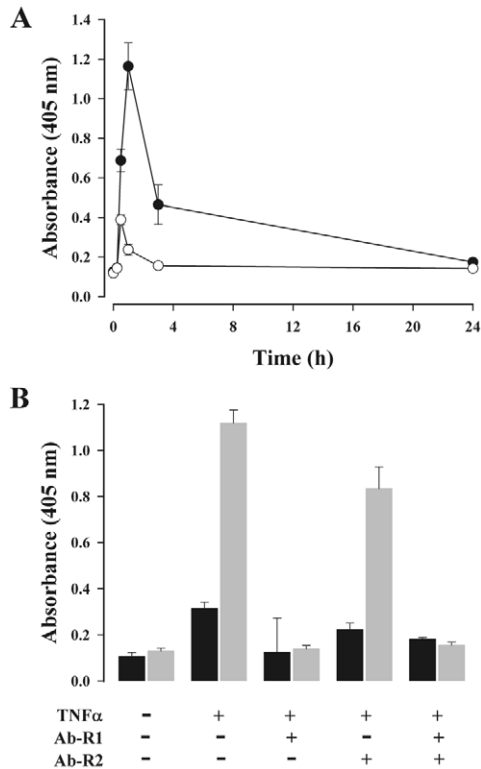
Fig. 8. TNFα induces activation of caspase 8. (A) Time course of activation of caspase 8 in LNCaP cells infected with Ad-Control (○) or Ad-Cx43 (●) after treatment with 10 ng/ml TNFα for variable lengths of time. Caspase 8 activity was significantly greater in the Ad-Cx43-infected cells as compared to those infected with control virus at 30 minutes, 1 hour and 3 hours ($P<0.01$). (B) Bar graph showing the effects of pre-treatment with TNFR neutralizing antibodies (10 µg/ml) upon the TNFα-induced caspase 8 activation in LNCaP cells infected with Ad-Control (black bars) or Ad-Cx43 (gray bars). Ab-R1 and Ab-R2 indicate antibodies directed against TNFR1 and TNFR2, respectively. Ad-Control-infected LNCaP cells showed a small increase in caspase 8 activity that was abolished by pretreatment with anti-TNFR1 antibodies ($P=0.0183$ for Ad-Control + TNFα vs Ad-Control alone; $P=0.0381$ for Ad-Control + TNFα vs Ad-Control + TNFα + Ab-R1; and not significant for Ad-Control + TNFα + Ab-R1 vs Ad-Control alone). Ad-Cx43-infected cells showed a much larger increase in caspase 8 activity that was abolished by pretreatment with anti-TNFR1 antibodies or anti-TNFR1 plus anti-TNFR2 antibodies ($P<0.0001$ for Ad-Cx43 + TNFα vs Ad-Cx43 alone or Ad-Cx43 + TNFα +Ab-R1 or Ad-Cx43 + TNFα +Ab-R1 +Ab-R2; Ad-Cx43 + TNFα +Ab-R1 or Ad-Cx43 + TNFα +Ab-R1 +Ab-R2 did not significantly differ from Ad-Cx43 alone). Pretreatment of Ad-Cx43-infected cells with anti-TNFR2 antibodies partially antagonized the TNFα-induced activation of caspase 8, since it significantly reduced the caspase 8 activity as compared to Ad-Cx43-infected cells treated with TNFα ($P=0.0009$), but levels were still much higher than those in untreated Cx43-expressing cells ($P<0.0001$).

after 10-12 days in culture (Mehta et al., 1999). Tanaka and Grossman found that infection of LNCaP cells with a recombinant Cx26-adenovirus alone caused growth inhibition, an effect that they attributed to reduced levels of Bcl-2 protein (Tanaka and Grossman, 2004). The difference between their results and ours in the same cell type must reflect connexin

type-specific effects (not produced by expression of Cx37, Cx40 or Cx43); in preliminary studies, we have found no change in Bcl-2 mRNA expression or protein levels in response to infection of LNCaP cells with Ad-Cx43 (unpublished observation).

Since connexins have been shown to form 'hemi-channels' linking the cytoplasm and extracellular milieu in single cells, the connexin-dependent function responsible for augmenting killing of LNCaP cells could have been mediated by either hemi- or complete gap junction channels. Indeed, several studies have implicated hemichannels in cell survival and death (Contreras et al., 2004; Hur et al., 2003; Plotkin et al., 2002). However, our cell density experiments suggest the involvement of full channels, since the LNCaP cells exhibited much more TNFα-induced cytotoxicity when cultured at high density (favoring cell contact-dependent phenomena) than when cultured at lower densities.

Several lines of evidence show a correlation between the ability of Ad-Cx43 infection to increase intercellular communication and its ability to enhance TNFα-dependent apoptosis. (1) No increased killing was seen in PC3 cells infected with Ad-Cx43, even though those cells produced detectable Cx43 protein. As previously reported (Govindarajan et al., 2002) and confirmed in our studies, introduction of Cx43 does not lead to production of gap junction plaques or an increase in intercellular communication in PC3 cells. (2) Expression of the non-functional Cx43DN did not enhance TNFα killing of LNCaP cells. (3) TNFα-induced killing of LNCaP cells was blocked by co-infection with Ad-Cx43DN (which also blocked any increase in intercellular communication). (4) The severity of the TNFα-induced effect on Cx43-expressing LNCaP cells correlated with cell density. Taken together, our data support the conclusion that the killing of Ad-Cx43-infected LNCaP cells by TNFα was due to increased intercellular communication. However, they contrast with previous data from other cells suggesting that induction of apoptosis by different inducers was influenced by properties of Cx43 other than gap junction function (Huang et al., 2001). For example, expression of the C-terminal domain of Cx43 suppresses the growth of Neuro2A (Moorby and Patel, 2001) and HeLa cells (Dang et al., 2003). Moreover, retrovirally-delivered Cx26 suppresses the growth of breast cancer tumors in mice without restoring intercellular communication (Qin et al., 2002).

The intrinsic pathway of apoptosis can be triggered by a number of stimuli that generate small molecules or ions that could permeate through gap junction channels. In the case of suicide gene therapy, toxic small molecules such as ganciclovir metabolites are produced (Colombo et al., 1995; Fick et al., 1995; Mesnil et al., 1996). Many apoptotic stimuli produce Ca^{2+} fluxes and involve inositol trisphosphate (IP_3) receptor signaling (Hanson et al., 2004; Orrenius et al., 2003). Calcium ions act as intercellular death messengers among ischemic astrocytes (Budd and Lipton, 1998; Lin et al., 1998) and among bladder cancer cells (Krutovskikh et al., 2002). Altered regulation of ion channels and cytoplasmic concentrations of Ca^{2+}, K^+, and/or Cl^- occur in both cell proliferation and cell death (Lang et al., 2005). Therefore, facilitation of intercellular communication through increased connexin expression might potentiate the intrinsic pathway of apoptosis as suggested previously. For example, Tanaka and Grossman found that infection with a Cx26 adenovirus augmented suppression of the growth of PC3 and

DU-145 cells by doxorubicin (Tanaka and Grossman, 2004). Moreover, in both head and neck cancer cells (Frank et al., 2005) and Cx32-transfected BHK cells (Udawatte and Ripps, 2005), bystander apoptosis can be mimicked by cellular loading or direct microinjection of cytochrome C, which is followed by death of both the loaded cell and its neighbors. Our data contrast with these previous observations, since we saw no connexin-dependent enhancement of killing by three agents that lead to apoptosis through the intrinsic pathway: etoposide, doxorubicin or staurosporine. The different outcome of our experiments probably reflects cell-type specific differences in signaling pathways or susceptibility to the agents tested.

Our data showed the unique finding that Cx43 expression enhanced the killing of LNCaP cells by TNFα, TRAIL and anti-Fas antibodies. Because these agonists of three different members of the TNF receptor superfamily had similar effects, it is possible that Cx43 expression provides a general sensitization of LNCaP cells to activation of the extrinsic pathway of apoptosis. The enhancement of TNFα-induced apoptosis in LNCaP cells infected with Ad-Cx43 was dependent on intercellular communication without altering TNFR levels; thus, it is likely that the sensitization was mediated through intercellular transfer of small molecules or ions involved in this pathway. Several studies have shown that Ca^{2+} and IP_3 are critical mediators of extrinsic cell death pathways (Boehning et al., 2005; Jayaraman and Marks, 1997), and in some cells, cAMP levels modulate TNFα induced apoptosis (Guevara Patino et al., 2000; Niwa et al., 1999). Alternatively, it is also conceivable that increased abundance of Cx43 in the plasma membrane might exert local effects (either directly or indirectly through association with accessory proteins) that enhance the association of TNF receptors with each other or with death-domain containing proteins.

Although a number of possible gap junction permeant death and survival signals have previously been suggested including IP_3, cAMP and Ca^{2+} ions (Krysko et al., 2005), the number of likely candidates for the effects upon LNCaP cells is narrowed by the selective ability of Cx43, but not Cx37 or Cx40, to sensitize these cells to apoptosis. Studies of the relative permeabilities of Cx37 and Cx40 channels to fluorescent dyes (including fluorescein and Alexa derivatives) and their ionic selectivities have shown that they are less permeable to larger, negatively charged ions and molecules than Cx43 channels (Beblo et al., 1995; Veenstra et al., 1995; Weber et al., 2004). Consistently, we found that whereas infection with Ad-Cx43 allowed Lucifer Yellow (M_r 457, net charge –2) transfer among LNCaP cells, expression of Cx37 or Cx40 allowed intercellular passage of neurobiotin (M_r 345, net charge +1), but not Lucifer Yellow. Since expression of Cx37 or Cx40 did not facilitate killing by TNFα, the permeant signals responsible for the effects observed in the current study might be molecules that selectively permeate Cx43 channels (such as IP_3 or cyclic nucleotides, which are negatively charged and have a similar size to Lucifer Yellow). The intercellular permeant molecules need not directly serve as 'death signals'; they might also have effects on signaling pathways downstream of TNFR1.

Whereas early or localized prostate cancer is potentially curable, there are limited treatment options available for advanced stages of the disease because of the ineffectiveness of chemotherapy and radiotherapy or the development of metastases. Defects in apoptotic signaling pathways are

common in cancer cells, and they probably are involved in the initiation and progression of prostate cancer and its resistance to various forms of therapy (Uzgare and Isaacs, 2005). Many cancer cells are also characterized by dysfunction of intercellular communication as well as apoptosis (Mesnil et al., 2005). Therefore, effective enhancement of apoptosis as seen with expression of Cx43 in LNCaP cells is a reasonable approach for developing more successful treatments.

Materials and Methods
Chemicals
Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. (St Louis, MO, USA). Recombinant human TNF-related apoptosis-inducing ligand (TRAIL) was obtained from PeproTech, Inc. (Rocky Hill, NJ, USA).

Connexin adenoviruses
The generation of recombinant adenoviruses containing rat Cx43 (Ad-Cx43), rat Cx40 (Ad-Cx40), human Cx37 containing a FLAG epitope appended to its carboxyl terminus (Ad-Cx37), rat Cx43 with a deletion of amino acids 130-136 and a C-terminal HA epitope tag (Ad-Cx43DN), and a control adenovirus with no gene insert following the CMV promoter (Ad-Control) has recently been described (Seul et al., 2004; Wang et al., 2005). The viruses were purified using double cesium chloride gradients, and viral titers were determined using the Adeno-X Rapid Titer Kit (BD Biosciences Clontech, Palo Alto, CA, USA). In all experiments, viruses were used at an MOI of 20:1; at this ratio of infection with the recombinant adenoviruses, all cells express connexin as assessed by immunofluorescence.

Cell culture
Prostate cancer cell lines, LNCaP and PC3, were obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were grown in RPMI 1640, containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate, in a humidified 5% CO_2 atmosphere at 37°C.

Antibodies
Anti-Fas monoclonal antibody (clone CH11) was obtained from Upstate Biologicals (Lake Placid, NY, USA). Neutralizing anti-TNFα receptor 1 (TNFR1) and anti-TNFα receptor 2 (TNFR2) antibodies were purchased from R & D Systems (Minneapolis, MN, USA). Immunodetection of Cx43, Cx37 and Cx40 was performed using rabbit polyclonal antibodies directed against a bacterially expressed fusion protein containing the carboxyl terminus of Cx43 (Sigma), a mouse monoclonal anti-FLAG antibody (Sigma), and rabbit polyclonal antibodies directed against a synthetic peptide immunogen corresponding to residues 316-329 of Cx40 (Kanter et al., 1993), respectively. Rabbit polyclonal anti-HA antibodies and mouse monoclonal antibodies directed against the cytoplasmic loop of Cx43 (anti-Cx43IL) and against the Golgi 58K protein (clone 58K-9) were obtained from Sigma. A mouse monoclonal anti-protein-disulfide isomerase (PDI) antibody was purchased from Affinity Bioreagents (Golden, CO, USA). Antibodies directed against β-actin were obtained from Cell Signaling (Beverly, MA, USA). Cy2- and Cy3-conjugated goat anti-rabbit or anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch (West Grove, PA, USA).

Cell viability assay
Cell viability was measured using the CellTiter 96 AQ nonradioactive cell proliferation assay (MTS; Promega, Madison, WI, USA). Cells were plated on 96-well plates at a density of 2000 cells/well in 90 μl of medium. After 48 hours, cells were infected with adenoviruses and incubated for an additional 12 hours. Then, cells were treated with TNFα, etoposide, doxorubicin, staurosporine, anti-Fas antibody or TRAIL for another 48 hours. After that time, MTS solution was added (20 μl/well), and cells were incubated for 1 hour at 37°C in a humidified 5% CO_2 atmosphere, the absorbance at 490 nm was measured with a microplate reader. Three independent experiments were performed in triplicate.

In some experiments, neutralizing anti-TNFR1 and/or anti-TNFR2 antibodies were added to the culture medium 2 hours prior to addition of TNFα.

To examine the effects of varying cell densities, LNCaP cells were plated on 96-well trays at different densities (1250, 2500, 5000 or 10,000 initial cells/well in 90 μl of culture medium). After 12 hours (when cells initially plated at 10,000 cells per well had reached 70% confluence), cells were infected with Ad-Cx43 and incubated for an additional 12 hours. Then, cells were treated with different concentrations of TNFα for another 48 hours. After that time, cell viability was measured using the MTS assay as described above. Three independent experiments were performed in triplicate.

Immunofluorescent labeling of cells
PC3 or LNCaP cells were plated on four-well tissue culture Lab-Tek chamber slides

(Nalge Nunc International, Naperville, IL). When cells reached 70% confluence, they were infected with adenovirus and incubated for another 12 hours. Cells were fixed with 4% paraformaldehyde and permeabilized with 1% Triton X-100. For single staining experiments, cells were reacted with rabbit anti-Cx43 or anti-Cx40 antibodies or with a mouse monoclonal anti-FLAG antibody (to detect Cx37) followed by Cy2-conjugated goat anti-rabbit or anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA) as appropriate.

For double-labeling analysis of cells that had been co-infected with Ad-Cx43 and Ad-Cx43DN, fixed cells were incubated simultaneously with a mouse anti-Cx43IL antibody (which recognizes the wild-type Cx43, but not Cx43DN) and rabbit polyclonal anti-HA antibodies (to detect Cx43DN). Double label detection of Cx43DN and ER or Golgi resident proteins was performed using fixed cells that had been infected with Ad-Cx43DN, using rabbit anti-Cx43 antibodies and mouse monoclonal anti-PDI or anti-Golgi 58K protein antibodies. Then, cells were incubated with Cy2- and Cy3-conjugated secondary antibodies (Jackson ImmunoResearch).

Photomicrographs were obtained using a Zeiss Axioplan 2 microscope equipped with a mercury lamp and an Axiocam digital camera using Zeiss AxioVision software (Carl Zeiss, München, Germany).

Immunoblotting

Cells were harvested in PBS. Protein concentrations were determined by the method of Bradford (Bradford, 1976) using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Equal amounts of protein (20 μg/lane for Cx43 and 100 μg/lane for TNFR and β-actin) were resolved by electrophoresis through 10% SDS-containing polyacrylamide gels, transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA), and incubated with rabbit anti-Cx43, anti-TNFR, or anti-β-actin antibodies, followed by peroxidase-conjugated goat anti-rabbit IgG or donkey anti-mouse IgG antibodies (Jackson ImmunoResearch). Binding of secondary antibodies was detected using enhanced chemiluminescence (ECL; Amersham, Arlington Heights, IL).

Transfer of microinjected tracers

LNCaP or PC3 cells were cultured on glass coverslips and infected with adenoviruses or left uninfected. Twenty four hours later, intercellular coupling was assessed after microinjection of 5% Lucifer Yellow with or without 4% neurobiotin (Vector Laboratories, Burlingane, CA) into one cell of a cluster. The extent of intercellular transfer of Lucifer Yellow was determined by counting the number of cells containing the tracer (excluding the injected cell) 2 minutes after the microinjection. The number of cells to which neurobiotin transferred was determined after fixing the cells in 4% paraformaldehyde followed by incubation with streptavidin–Cy3 conjugate (Martinez et al., 2002).

Assessment of apoptosis by flow cytometry

Approximately 5×10^4 LNCaP cells were plated on 60 mm dishes and cultured for 48 hours (about 70% confluence). Then, cells were infected with adenovirus and incubated for 12 hours. Cells were treated with 10 ng/ml of TNFα and incubated for another 12 hours (for annexin V labeling) or 48 hours (for cell cycle analysis). The cell culture medium containing floating cells was collected. Attached cells were rinsed with Ca^{2+}- and Mg^{2+}-free PBS and harvested after incubation with 2 mM EDTA in PBS for 10 minutes. Floating and attached cells were pooled and rinsed twice with PBS.

Annexin V/propidium iodide labeling

Cells were resuspended in $1\times$ binding buffer and incubated with FITC-conjugated annexin V and propidium iodide (PI) for 15 minutes at room temperature in the dark using the FITC-annexin V apoptosis detection kit (BD Biosciences, San Diego, CA). The samples were immediately analyzed on a FACScalibur flow cytometer (Becton Dickinson Immunocytometry System, San Jose, CA) using the CellQuest Pro Software.

Cell cycle analysis

Cells were fixed with 70% ethanol and stained with PI (50 μg/ml) in the presence of RNAse A (100 U/ml). PI-stained cells were detected with the FL-2 photomultiplier of the FACScalibur flow cytometer. The percentage distribution of cells in the different phases of the cell cycle was determined. The fraction of apoptotic cells was quantified by analysis of the sub-G1 peak (sub-diploid cells) (Ormerod et al., 1992). Three independent experiments were performed for each assay.

Caspase 8 activity assay

LNCaP cells plated on six-well dishes were allowed to reach 70% confluence. Cells were infected with Ad-Control or Ad-Cx43. Twelve hours later, they were treated with 10 ng/ml of TNFα for different time intervals. Then, cells were harvested and lysed, and the protein concentrations were determined. Caspase 8 activity was assayed using a caspase 8 colorimetric activity assay kit (Chemicon, San Diego, CA, USA). Absorbance at 405 nm was measured using a microplate reader. In some experiments, TNFR neutralizing antibodies (10 μg/ml) were added 2 hours before treatment with TNFα.

Statistics

Statistical significance for the intercellular transfer data was evaluated using Student's *t*-test. Cell viability and caspase 8 activity data were analyzed using Tukey-adjusted pairwise comparisons. Mixed effects models were constructed for all MTS assay and caspase 8 activity data. The models were reduced to ones of only main effects when the interaction term was not significant ($P \geqslant 0.05$). For the MTS data, all plotted values were normalized by dividing the raw value by the mean across all replications of no virus and no TNFα within each experiment. Standard errors of these ratios were computed using the delta method. For caspase 8 activity data, the A_{405} values were compared. All data shown are the mean of at least three independent experiments.

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