Adenoviral delivery of human connexin37 induces endothelial cell death through apoptosis

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Abstract

Gap junction channels formed of connexins directly link the cytoplasm of adjacent cells and have been implicated in intercellular signaling that may regulate the functions of vascular cells. To facilitate connexin manipulation and analysis of their roles in adult endothelial cells, we developed adenoviruses containing the vascular connexins (Cx37, Cx40, and Cx43). We infected cultured human umbilical vein endothelial cells with control or connexin adenoviruses. Connexin expression was verified by immunoblotting and immunofluorescence. Infection with the Cx37 adenovirus (but not control or other connexin adenoviruses) led to a dose-dependent death of the endothelial cells that was partially antagonized by the gap junction blocker α-glycyrrhetinic acid and altered the intercellular transfer of Lucifer yellow and neurobiotin. Cell morphology, Annexin V and TUNEL staining, and caspase 3 assays all implicated apoptosis in the cell death. These data suggest that connexin-specific alterations of intercellular communication may modulate endothelial cell growth and death.

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Gap junctions coordinate the activities of multiple cells within the walls of blood vessels. Gap junction channels allow intercellular passage of ions, second messengers (such as Ca\(^{2+}\), IP3, cAMP, and cGMP), and small metabolites. Endothelial cells contain at least three connexins; connexin37 (Cx37), connexin40 (Cx40), and connexin43 (Cx43), which are differentially expressed and distributed in fetal and adult blood vessels [1,2]. Cx40 is probably the most abundant component of adult endothelial cell gap junctions [3]; Cx43 may be most abundant at sites of turbulent flow [4]; Cx37 is abundantly expressed in the fetal vasculature, but it is down-regulated in most adult endothelial cells, except in pulmonary vessels [5]. Due to their channel properties and the regulation of expression during vascular development and in diseases such as wound healing [6], hypertension [7], and atherosclerosis [8], it has been suggested that gap junctions play a role in the growth and death of endothelial cells.

Cells within a microenvironment communicate with their neighbors to maintain population homeostasis by exchanging signals for survival [9] and for death [10–15]. Death signals can be endogenous ones (like calcium, cAMP or cGMP) [16], or exogenous ones (like ganciclovir triphosphate that modulates the bystander or “kiss of death” effect) [17]. These molecules can pass through gap junctions to induce cell death in neighboring healthy cells [11,15,17].

While disruption of Cx37, Cx40, and Cx43 genes individually has modest vascular effects, a recent knock-out study showed that mice null for both Cx37 and Cx40 exhibited defects of vascular endothelial development [18]. Our previous experiments show that Cx37 forms heteromeric channels with co-expressed Cx43, with biophysical properties that differ from those of either Cx37 or Cx43 homomeric channels [19]. Moreover, dye transfer experiments suggest that co-expression of these connexin may also change channel permeability [20].
However, the precise roles of the connexins in vascular tissue remodeling or diseases have not been elucidated, because there has not been an appropriate system for manipulating connexins in adult vascular tissues. We recently developed adenoviruses containing the vascular endothelial connexins, Cx37, Cx40 or Cx43.

We hypothesized that perturbation of interendothelial communication patterns by adenoviral delivery of different connexins might alter the balance of transfer of survival and death signals between cells. We tested this hypothesis in cultures of human umbilical vein endothelial cells (HUVEC) and other cells (normal rat kidney epithelial cells, NRK, and mouse neuroblastoma cells, N2A). Our data show that endothelial connexins (especially Cx37) may play critical roles in survival and death of endothelial cells.

Materials and methods

Connexin adenoviruses

DNA fragments containing the coding regions of human Cx37, mouse Cx40, and rat Cx43 were used to produce recombinant adenoviruses. The FLAG epitope (DYKDDDDK) was attached to the carboxyl terminus of the Cx37 coding region [20]. A control virus was constructed with no insert following the CMV promoter. Recombinant adenoviruses were generated with the AdEasy System (Quantum Biotechnologies, West Montreal, Canada) and purified using double cesium chloride gradients. Viral titers were determined using the Adeno-X Rapid Titer Kit (BD Biosciences Clontech, Palo Alto, CA). The titers (in infectious units, ifu) of viral stocks were determined by immunostaining-infected HEK293 cells after 48 h with antibodies directed against the viral hexon protein.

Cell culture

Human umbilical vein endothelial cells and media were purchased from Clonetics (Walkersville, MD). NRK and N2A cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum at 37 °C in an atmosphere of 5% CO2. HUVEC were used in passages two through five. Unless otherwise noted, cells were infected with virus about 12 h after achieving confluence. Virus was applied for 1 h and then cells were changed to fresh medium.

Immunofluorescence, immunoblot analysis, and RNA blots

Cx40 and Cx43 were detected using rabbit polyclonal antibodies directed against bacterial fusion proteins containing carboxy-terminal regions of these proteins [21,22]. Cx37 was detected using an antibody recognizing the FLAG epitope (Sigma, St. Louis, Missouri) as described previously. [20] For immunostaining, confluent HUVEC were cultured on gelatin-coated 4 chamber slides (Nalge Nunc, Naperville, IL) and infected with virus (0.9 × 106 ifu/cm² of culture dish). After 24 h, cells were fixed, permeabilized, and reacted with primary and Cy3-conjugated secondary antibodies to detect expressed connexins [20,21]. Immunoblots were performed using whole cell lysates [20,22] of confluent HUVEC grown in 100 mm culture dishes that were harvested 1 day following infection with control or Cx37 virus (1.8 × 106 ifu/cm² of culture dish). The cDNA probes contained coding sequences of human Cx37, mouse Cx40, or rat Cx43. Preparation, labeling of probes with 32P, and hybridization were performed with methods previously described [20]. Blots were exposed for autoradiography with X-ray film or a phosphor screen. Images from the exposed phosphor screen were scanned, and radioactivity was quantified by computer imaging (PhosphoImager with ImageQuant software; Molecular Dynamics, Sunnyvale, CA).

Viability assay

Cells were seeded into 24-well culture dishes (2 × 10⁵ cells/well). Cells were infected with viruses after overnight culture. Cell viability was determined colorimetrically after 48 h using the XTT assay (Roche Diagnostics, Indianapolis, Indiana), normalized to the untreated values, and expressed as the mean ± SEM from triplicate determinations.

Modification of Cx37-induced endothelial cell death

To block gap junction channels, 18 μM of 18α-glycyrrhetinic acid (Sigma, St. Louis, Missouri) was applied to HUVEC 4 h before infection with Cx37- or control adenovirus. Three doses of virus, 2, 4, and 8 × 10⁶ ifu/cm² of culture dish were tested. Cell viability was not affected by any of the doses of control viruses; therefore, data obtained using the highest dose were used for comparison to the Cx37 virus-infected HUVEC (Fig. 4A). To clarify the effect of down-regulated endogenous Cx43 on the Cx37-induced cell death, Cx43 adenovirus (3.6 × 10⁶ ifu/cm² of culture dish) and Cx37 adenovirus (1.8 × 10⁶ ifu/cm² of culture dish) were added simultaneously to HUVEC cultures. Cell viability was determined colorimetrically 48 h after adenovirus treatment using the XTT assay (Roche Diagnostics, Indianapolis, Indiana), normalized to the untreated values, and expressed as the mean ± SEM for triplicate determinations.

Microinjection of gap junction tracers

Intercellular communication was assessed by transfer of microinjected Lucifer yellow and neurobiotin using a minor modification of previous methods [23]. HUVEC in 60 mm culture dishes were infected with control or Cx37 virus (1.8 × 10⁶ ifu/cm² of culture dish). After 30 h, cells were impaled with a micropipette filled with 150 mmol/L LiCl, 4% Lucifer yellow (charge = -2, molecular weight 457; Sigma, St. Louis, Missouri), and 4% neurobiotin (charge = +1, molecular weight 322.8; Vector Laboratories, Burlingame, CA). Solutions were microinjected with a picopump (model PLI-188, Nikon) by using 0.2- to 0.3-s pulses of 1–2 psi; cells were impaled for 0.5–1 min. After the microinjection, the cells were fixed with paraformaldehyde (4% in PBS) for 30 min and then permeabilized with methanol/acetone (1:1) for 2 min at room temperature. The neurobiotin tracer was detected after staining the cells with streptavidin–Cy3 conjugate (Sigma, St. Louis, Missouri). The extent of intercellular transfer of both tracers was determined by recording the number of adjacent cells containing the tracer after visualization by epifluorescence and digital microscopy. More than 18 cells were injected in each group, and results were compared using Student’s t test.

Apoptosis assays

Annexin V and propidium iodide staining. Confluent HUVEC cultured on gelatin-coated, 4 chamber slides were infected with control or Cx37 virus (1.8 × 10⁶ ifu/cm² of culture dish). After 36 h, Annexin V binding was visualized using the Annexin-V-Fluos staining kit (Roche Diagnostics, Indianapolis, Indiana). Cells which had lost membrane integrity were identified by propidium iodide (PI) staining without detergent permeabilization.

Caspase 3 assay. HUVEC in 100 mm culture dishes were infected with control or Cx37 virus (1.8 × 10⁶ ifu/cm² of culture dish). After 36 h, cells were harvested, protein concentrations were determined, and caspase 3 activity was measured using a colorimetric assay (R&D Systems, Minneapolis, New England).

TdT-mediated dUTP nick-end labeling (TUNEL) assay. HUVEC were cultured on gelatin-coated 4 chamber slides and treated with...
control- or Cx37 virus (1.8 × 10^6 ifu/cm^2 of culture dish). Fragmented DNA was detected using the DeadEnd Fluorometric TUNEL system (Promega, Madison, Wisconsin), and all cells were stained with PI after membrane permeabilization.

Results

Expression and localization of connexins delivered by adenoviruses

We tested expression of vascular connexins using cultured human umbilical vein endothelial cells. Immunoblot and RNA blot analysis showed that Cx43 was expressed endogenously by these cells, as previously shown [1,2]. Cx40 was not detected by either immunoblot or RNA blot. Cx37 mRNA was weakly detectable by RNA blotting in both control virus infected (Fig. 1C) and uninfected HUVEC (data not shown). After infection with Cx37 and Cx40 adenoviruses, the HUVEC showed specific immunoreactivity that localized to appositional membranes as expected for gap junction proteins (Fig. 1A, lower panels). The ability of Cx43 viruses to produce connexin proteins was verified by infection of other cell types that do not endogenously produce this protein (not shown). Immunoblots and RNA blots also confirmed production of connexin proteins in connexin adenovirus-infected HUVEC (Figs. 1B and C). The abundance of Cx37 protein produced in response to Cx37 adenovirus infection increased with increasing viral doses (Fig. 1B, right panel). Infection with control virus had no effect on the abundance of endogenous Cx43 (Fig. 1B, left panel). The abundance of endogenous Cx43 mRNA was not changed in RNA blots of Cx37 adenovirus-infected cells (Fig. 1C, middle), but endogenous Cx43 protein levels were dramatically reduced.

![Fig. 1. Expression of connexins in cultured endothelial cells following adenoviral infection.](image-url)

(A) Immunofluorescent detection of connexins in HUVEC 24 h after infection with control viruses (top panels) or with Cx37, Cx43, or Cx40 adenoviruses (lower panels). These cultured human endothelial cells endogenously expressed Cx43, but not the other connexins. In contrast, HUVEC infected with the connexin adenoviruses showed abundant staining between cells corresponding to each connexin. Bar, 10 μm. (B) Immunoblotting. Cell lysates were harvested from HUVEC 24 h after infection with different doses (ifu/cm^2 of culture dish) of control or Cx37 adenoviruses. Immunoreactive Cx37 was not detected in control cells, but levels showed a dose-dependent increase in the Cx37 adenovirus-infected cells. HUVEC infected with control virus showed consistent levels of Cx43, but cells infected with Cx37 adenovirus showed decreased Cx43 levels. (C) RNA blots hybridized with Cx37, Cx43 or Cx40 probes using RNA isolated from HUVEC 24 h after infection with control or Cx37 viruses.
Impact of connexins on cell growth and viability

Infection of HUVEC with Cx37 adenovirus produced extensive cell death within the cultures (Fig. 2A). To quantitate this observation, we used a colorimetric viability assay to examine the effects of control and connexin adenoviruses on HUVEC and two cell lines from different sources (NRK cells which are well coupled and express Cx43 and N2A cells which do not express connexins and are communication-deficient) (Fig. 2A). Forty-eight hours after infection of confluent HUVEC monolayers with Cx37 adenovirus, cell viability was reduced to less than 20% of controls. In contrast, Cx43 and Cx40 adenoviruses did not kill

Fig. 2. Effects of connexins on cell viability. (A) Connexin and cell type specificity of the effects of connexin adenoviruses. Confluent cultures of three different cell types (HUVEC, NRK, and N2A) were infected with control or connexin adenoviruses (0.9 \times 10^6 ifu/cm^2 of culture dish) and cell viability was determined after 48 h by the colorimetric XTT assay. *P < 0.001. (B) Dose dependence of Cx37-induced endothelial cell death. Confluent HUVEC cultures were infected with different doses of Cx37- or control adenoviruses and cell viability was quantitated at 48 h using the XTT assay.

Fig. 3. Modulation of Cx37-induced cell death. (A) Effects of gap junction blockade. Confluent cultures of HUVEC were pre-treated for 4 h with 18 \mu M 18\alpha-glycyrrhetinic acid (GA) or vehicle alone prior to infection with control adenovirus or two different doses of Cx37 adenovirus. Cell viability was determined after 48 h using the XTT assay. Asterisks indicate values that differed between GA and control treatments (P < 0.001). (B) Effects of co-expression of Cx43. Confluent cultures of HUVEC were infected with control, Cx37 or both Cx37 and Cx43 adenoviruses. After 48 h, connexin proteins were detected by immunoblotting (top), and cell viability was quantitated by XTT assays. *Compared with control group (P < 0.001). #Compared with Cx37 group (P < 0.001).
HUVEC. Infection of NRK or N2A cells with comparable doses of the connexin adenoviruses had no significant effects. Taken together, our data suggest that the connexin adenoviruses had connexin and cell type specific effects on cell viability. We further investigated the Cx37 effects using different doses of virus (Fig. 2B). While HUVEC viability was minimally affected by infection with different doses of control virus, cell viability fell dramatically with increasing doses of Cx37 adenovirus.

**Interventions to modify Cx37-induced endothelial cell death**

To confirm the role of intercellular communication in the cell death that followed infection with the Cx37 adenovirus, we pretreated HUVEC cultures with the gap junction blocker, 18α-glycyrrhetinic acid, and then infected them with doses of Cx37 adenoviruses that should cause intermediate levels of cell death. The cell viability was partially, but significantly, increased (Fig. 3A), implicating gap junction channel function in the Cx37-induced endothelial cell death. In contrast, application of conditioned medium from Cx37 adenovirus-infected HUVEC cultures to uninfected cells had no effect on cell viability (not shown), suggesting that the death signals did not travel through an extracellular route. Although endogenous Cx43 levels were decreased by infection with Cx37 adenovirus, simultaneous infection with Cx43 adenovirus elevated Cx43 levels, but did not prevent cell death (Fig. 3B).

**Changes of intercellular molecule transfer by Cx37 adenovirus infection**

To examine possible changes of intercellular communication, we examined intercellular passage of Lucifer yellow and neurobiotin in HUVEC within 30 h after infection with control or Cx37 adenoviruses (1.8 × 10^6 ifu/cm^2 of culture dish). Individual cells within a monolayer were microinjected with a mixture of both molecules, and the extent of gap junctional coupling was assessed by counting dye-filled neighbors. Intercellular transfer of Lucifer yellow to neighboring cells was decreased by Cx37 adenovirus infection, while neurobiotin transfer was increased in Cx37 adenovirus-infected cells (Figs. 4A and B). The relative numbers of cells receiving the two dyes (expressed as a ratio) changed dramatically (Fig. 4C).

**Cx37 kills HUVEC by inducing apoptosis**

To examine how Cx37 induced endothelial cell death, we performed several assays to detect activation of apoptosis. Confluent HUVEC cell cultures were infected with control or Cx37 adenoviruses, and they were examined through different time points when cell death was evident. Nuclear labeling of permeabilized cells with propidium iodide (PI) was performed (Fig. 5A). Cx37 adenovirus infection of confluent HUVEC monolayers led to the appearance of many rounded cells within about 1.5 days, followed by progressively increasing numbers of dying cells and detachment of cells from the culture plate. In HUVEC infected with comparable doses of control, Cx40, or Cx43 viruses, the cells maintained intact endothelial cell morphology.

An early event in apoptosis is the loss of membrane lipid asymmetry. Many of the cells in Cx37 adenovirus-infected (but not control) cultures showed extensive binding of FITC-conjugated annexin V, suggesting external exposure of phosphatidylserine which is normally present only in the inner face of the plasma membrane (Fig. 5B). These cells also showed extensive nuclear labeling with propidium iodide (PI) without any intervention to permeabilize the cells; HUVEC infected with control virus showed little PI labeling (Fig. 5B). Caspase 3 is an intracellular protease that is activated early during apoptosis and initiates cellular breakdown by degrading specific structural, regulatory, and DNA
repair proteins. We observed significantly greater levels of caspase 3 activity in Cx37-treated cells than in control virus-infected cells (Fig. 5C). We examined the time course of nuclear DNA fragmentation morphologically by performing a TUNEL assay at sequential time points following Cx37 or control adenovirus infection of HUVEC. The Cx37-treated cells showed numerous TUNEL positive cells beginning at 22 h with increasing numbers at subsequent time points, whereas no positive cells were seen in the control cultures. PI staining of detergent permeabilized cells at 48 h after infection with control virus demonstrated normal nuclear morphology, in contrast to extensive cell death and nuclear fragmentation in Cx37 virus-infected HUVEC (Fig. 5D).

Discussion

Our data have demonstrated that altered expression of connexins in endothelial cells due to adenoviral delivery of Cx37 resulted in cell death by inducing apoptosis. While it may seem surprising that expression of a molecule that mediates intercellular communication would lead to apoptosis in adult endothelial cells, a number of recent studies of cultured cell lines have implicated gap junctions in affecting cell survival, proliferation, and death suggesting possible mechanisms. While many death inducers (including Fas ligand, tumor necrosis factor α, growth factor depletion, and DNA damaging agents) come from the outside of cells, individual epithelial and endothelial cells may also spontaneously commit suicide [10]. Interacting cell neighbors can exchange signals for survival [9] or death [14,15]. Many agents that mediate death signals are intercellularly permeable, including endogenous molecules (e.g., calcium, cAMP, and cGMP) [16,17] and exogenous ones (e.g., ganciclovir metabolites produced in thymidine kinase expressing cells mediating “bystander” cytotoxicity) [17,24]. Thus, generation of signaling molecules in one cell might induce apoptosis in neighboring healthy cells via passage through gap junction channels. If the connexins have different permeabilities for intracellular
death or survival signals, their effects on cell growth and death should be connexin-specific.

Our data show that the induction of apoptotic cell death was connexin-specific; it occurred after infection with Cx37 adenovirus, but not Cx40 or Cx43 adenoviruses. Moreover, the Cx37 effect must dominate, since the HUVEC endogenously express another connexin (Cx43). Cx37 has some properties that differ quantitatively from those of other connexins. Cx37 channels are among the largest conductance of any connexin channels, but they have limited permeability to some anions and dye tracers [17,25,26]. Cx37 forms heteromeric channels with co-expressed connexins such as Cx43, and these channels have conductance and permeability properties that differ from those of the homomeric channels [19,20]. Our dye injection experiments (Fig. 4) suggest that Cx37 expression (and perhaps the reduced Cx43 protein production) were associated with increased communication of one tracer (neurobiotin) but decreased passage of a second (Lucifer yellow) suggesting maintenance (or an increase) in cellular coupling, but a change in permeability. In parallel, the channel preferences for intracellular molecules might have shifted from survival signals toward death signals when Cx37 expression altered the gap junction channel permeability. Thus, expression of Cx37 might uniquely facilitate the passage of death signals, or it might prevent the passage of survival signals. Interestingly, Andrade-Rozental et al. [17] have suggested that the extent of cell death mediated by the thymidine kinase/ganciclovir bystander effect depends on connexin type and abundance.

The Cx37 cell death also was cell type specific. While infection with Cx37 adenoviruses killed endothelial cells, two other types of cells were tested, and no effects were observed. The endothelial cells may have unique intracellular signaling pathways that are more likely to spontaneously generate a death signal that can be preferentially passed through Cx37 channels.

The cell death that followed infection with the Cx37 adenovirus was likely related to the function of Cx37 as an intercellular channel protein. Glycyrrhetinic acid partially inhibited the effect at a dose that blocks intercellular dye coupling. Conditioned medium could not reproduce the effect, suggesting that it was not due to secretion of a mediator. Cx37 can form functional hemichannels [27], but the high calcium concentration present extracellularly in the culture medium would have prevented their opening. Augmented Cx37 expression may have altered the normal cellular coupling in these cells, since it was accompanied by a decrease in the levels of the endogenous Cx43. Endogenous Cx43 was down-regulated by exogenous Cx37, suggesting that this might contribute to initiation of cell death. To test this hypothesis, we overexpressed Cx37 in HUVEC with Cx43 adenovirus. Overexpressed Cx43 did not block Cx37-induced cell death (perhaps it was exacerbated), suggesting that the effect of Cx37 on the induction of endothelial cell death was dominant over Cx43. It is also possible that functions of Cx37 other than as a channel may play a role in cell death. It had been reported that Cx43-induced death of osteoblasts [28] and the anti-death activity of Cx43 in a glioma cell line are not related to channel function [29].

Our data demonstrate that intercellular communication through gap junctions is important in homeostasis of cell growth and death in primary adult endothelial cells. This strongly suggests that regulation of vascular connexins is important in tissue remodeling in vascular development and diseases.

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