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RESEARCH ARTICLE

Connexin 26 enhances the bystander effect in HSVtk/GCV gene therapy for human bladder cancer by adenovirus/PLL/DNA gene delivery

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Herpes simplex thymidine kinase/ganciclovir (HSVtk/GCV) gene therapy has been used for the treatment of a variety of cancers. Its efficacy is enhanced by the bystander effect that helps overcome the delivery problems commonly observed in current gene therapy. Connexins encode proteins that produce gap junctions, which enable intercellular communication and the bystander effect. We previously demonstrated that decreased Cx 26 expression and loss of gap junctional intercellular communication were associated with human bladder cancer. To investigate the efficacy of the bystander effect in HSVtk/GCV gene therapy, the Cx 26 gene was introduced into UM-UC-3 and UM-UC-14 bladder cancer cell lines by an adenovirus poly-L-lysine conjugate using a multigenic expression plasmid that expressed both the HSVtk and Cx 26 genes. We found significantly increased cytotoxicity in HSVtk/GCV gene therapy after introduction of the HSVtk and Cx 26 genes together compared with the cytotoxicity seen after introduction of the HSVtk gene and LacZ genes in vitro and in vivo. Cytotoxicity correlated with Cx 26 expression and the induction of functional gap junctions. This study indicates that combination gene therapy with co-expression of the HSVtk and Cx 26 genes potentiates HSVtk/GCV gene therapy through the bystander effect. Gene Therapy (2001) **8,** 139–148.

Keywords: *bladder cancer; connexin 26; adenovirus/PLL/DNA complexes; HSVtk/GCV gene therapy; bystander effect*

Introduction

Each year, more than 50000 new cases of bladder cancer are detected.[1] Superficial bladder cancers constitute 80% of these neoplasms and are associated with a high rate of tumor recurrence despite treatment consisting of transurethral resection and intravesical chemotherapy or immunotherapy.[2] Tumor progression within 5 years occurs in up to 30–40% of patients with high-risk superficial disease, and 15–34% of patients with superficial bladder cancer die of their cancer.[3,4] The prognosis for patients with advanced tumors is poor even for patients who receive aggressive multimodal therapy, with only 20–40% of patients with advanced bladder cancer surviving 5 years.[3,4] Thus, novel treatments are needed to improve the prognosis for patients with bladder cancer.

Connexins (Cxs) encode a family of transmembrane proteins known as gap junctional intercellular communication (GJIC) proteins, which mediate the transfer of ions, metabolites, and small regulatory molecules.[5] Cxs are involved in embryonic development, differentiation, and growth control.[6,7] In general, the expression of Cxs in human cancer cells is greatly reduced or undetectable. For example, reduced expression of Cx 43 in rat C6 glioma cells and of Cx 26 in human breast and bladder cancer cell lines is associated with decreased GJIC.[8–10] Conversely, transfection of Cx 43 in C6 glioma cells, Cx 32 in human liver cancer cells, and Cx 26 in HeLa cells and MCF7 breast cancer cells significantly reduces tumor cell growth *in vivo* and *in vitro*.[11–14]

Herpes simplex thymidine kinase/ganciclovir (HSV*tk*/GCV) gene therapy has been demonstrated to treat effectively various types of cancers *in vitro* and *in vivo*.[15–19] In HSV*tk*/GCV gene therapy, the toxicity of GCV is extended from HSV*tk*-transduced cells to adjacent HSV*tk*-negative cells through the so-called bystander effect. Some studies have shown that the bystander effect in HSV*tk*/GCV gene therapy is mediated by Cx expression and that the exogenous introduction of Cx 26 or Cx 43 enhances the bystander effect.[20–29] The bystander effect enhances the efficacy of HSV*tk*/GCV gene therapy by enabling toxic metabolites to pass to adjacent HSV*tk*-negative cells and helps overcome the current major limitation of gene therapy – the inability to achieve expression of the transgene in all cancer cells in a tumor.[30] The bystander effect has also been shown to facilitate other gene therapy approaches, such as suicide gene therapy involving transfer of the cytosine deaminase gene and tumor growth suppression involving the tumor suppressor gene *p53*.[31,32]

In this study, we investigated whether the Cx 26 gene increases the bystander effect in HSV*tk*/GCV gene ther-

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apy in human bladder cancer using an adenovirus poly-L-lysine (Adv/PLL) conjugate with a multigenic expression plasmid that expresses both the HSV*tk* and Cx 26 genes. Our data demonstrate the effectiveness of this construct in inducing the bystander effect in two human bladder cancer cell lines that do not express endogenous Cx 26.

Results

Transduction of bladder cancer cells in vitro

The transduction efficiencies of Adv/PLL/pGT60LacZ complexes were examined in UM-UC-3 and UM-UC-14 bladder cancer cells at various multiplicities of infection (MOIs) of viral particles per cell (Table 1). A high MOI was required to transduce X-gal because the MOI reflected the number of adenoviral particles and not plaque-forming units. Cytotoxicity was observed at MOIs that resulted in ⩾50% of cells expressing the *LacZ* gene.

The role of the Cx 26 gene in the bystander effect in vitro

To examine whether Cx 26 mediates the bystander effect, we infected UM-UC-3 and UM-UC-14 bladder cancer cells with Adv/PLL conjugate without plasmid DNA (control adenovirus), Adv/PLL/pGT60LacZ complexes, or Adv/PLL/pGT60hCx26 complexes at the indicated MOIs, or mock in the presence of culture medium containing 2% fetal bovine serum (FBS). Both Adv/PLL/pGT60LacZ and Adv/PLL/pGT60hCx26 contain the HSV*tk* gene (Figure 1). The degree of bystander effect-mediated cell killing was determined by measuring cell survival after 5 days of GCV treatment.

The cytotoxicity in cells infected with Adv/PLL/pGT60hCx26 complexes was significantly greater than that in cells infected with Adv/PLL/pGT60LacZ complexes (Figure 2). In comparison with control adenovirus-infected cells, UM-UC-3 cells infected with Adv/PLL/pGT60hCx26 complexes at 50 MOI exhibited growth inhibition of 76.6% and 80.4% at 10 and 50 μg/ml GCV, respectively, whereas cells infected with Adv/PLL/pGT60LacZ complexes showed only 6.5% and 37.9% growth inhibition, respectively (Figure 2a). In comparison with control adenovirus-infected cells, UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes at 100 MOI under the same condition also exhibited growth inhibition of 41.1% and 51.9% at 10 and 50 μg/ml GCV, respectively, whereas Adv/PLL/pGT60LacZ complexes showed growth inhibition of 6.7% and 25.1% (Figure 2b). The cytotoxicity produced by Adv/PLL/pGT60hCx26 complexes in UM-UC-3 and UM-UC-14 cells was statistically significant relative to the cytotoxicity produced by Adv/PLL/pGT60LacZ complexes at the same MOI and concentration of GCV ($P < 0.01$, Figure 2a, b). Control adenovirus infection resulted in a minor degree of growth inhibition that was significantly less than that seen after infection with Adv/PLL/pGT60hCx26 or Adv/PLL/pGT60LacZ complexes (Figure 2a, b). GCV was cytotoxic at a concentration as low as 10 μg/ml in UM-UC-3 and UM-UC-14 cells treated with Adv/PLL/pGT60hCx26 complexes but not in cells treated with Adv/PLL/pGT60LacZ complexes at intermediate and low MOIs (Figure 2a, b).

To examine the effect of Cx 26 as a tumor suppressor, we evaluated cell growth without the addition of GCV. Five days after infection, UM-UC-3 cells infected with Adv/PLL/pGT60hCx26 complexes at 50 and 100 MOI, although not at 25 MOI, showed a growth inhibition of 13% and 29.7%, respectively, compared with the growth of control adenovirus-infected cells ($P < 0.05$ at both MOIs, Figure 2c). The magnitude of growth suppression by Cx 26 alone was much less than that seen with Adv/PLL/pGT60hCx26 complexes in HSV*tk*/GCV gene therapy. There was no significant growth inhibition of UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes at 100 or 200 MOI (Figure 2d).

Table 1 The transduction efficiencies of the Adv/PLL/pGT60LacZ complexes in UM-UC-3 and UM-UC-14 bladder cancer cell lines *in vitro*

MOI	*UM-UC-3 Transduction efficiencies (%)/ toxicity*[a]	*UM-UC-14* Transduction efficiencies (%)/ toxicity[a]
25	8/–	1–2/–
50	14/±	6/–
100	22/+	12/±
200	46/++	28/+
500	66/+++	63/++

[a]The transduction efficiencies are presented as the percentage of blue-staining cells at different MOI at 48 h after incubation with the Adv/PLL/pGT60LacZ complexes. Four days after infection, toxicity was graded as follows: –, none; ±, weak; +, weak; ++, mild; +++, strong.

Detection of Cx 26 and HSVtk gene expression

We performed both Western and immunofluorescence analysis of UM-UC-3 and UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes. Western analysis demonstrated that Cx 26 expression in UM-UC-3 and UM-UC-14 cells was detected in all samples 2 days after infection with Adv/PLL/pGT60hCx26 complexes, but no Cx 26 expression was observed in the parental cells (Figure 3). In addition, Cx 26 protein expression was dose dependent, increasing when cells were infected at higher MOIs. Equal transduction efficiencies of both Adv/PLL/pGT60LacZ and Adv/PLL/pGThCx26 complexes at the same MOIs were observed, as seen by Western blot analysis of HSV*tk* gene expression in infected cells (Figure 2a, b). Immunofluorescence showed that UM-UC-3 and UM-UC-14 cells expressed Cx 26 2 days after infection with Adv/PLL/pGT60hCx26 complexes (Figure 4b, d), but mock-infected (data not shown) and cells infected with Adv/PLL/pGT60LacZ complexes at 50 and 100 MOI showed no expression (Figure 4a, c). Immunofluorescence of UM-UC-3 and UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes demonstrated a punctate staining pattern compatible with gap junction assembly (Figure 4b, d). We also observed the same pattern of Cx 26 expression in RT-4 human bladder cancer cells, which constitutively express Cx 26 and have functional gap junctions (data not shown).

Functional assay for GJIC of bladder cancer cells

We observed the presence of functional GJIC in UM-UC-3 and UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes using the scrape-loading lucifer yellow dye transfer assay. Two days after infection with

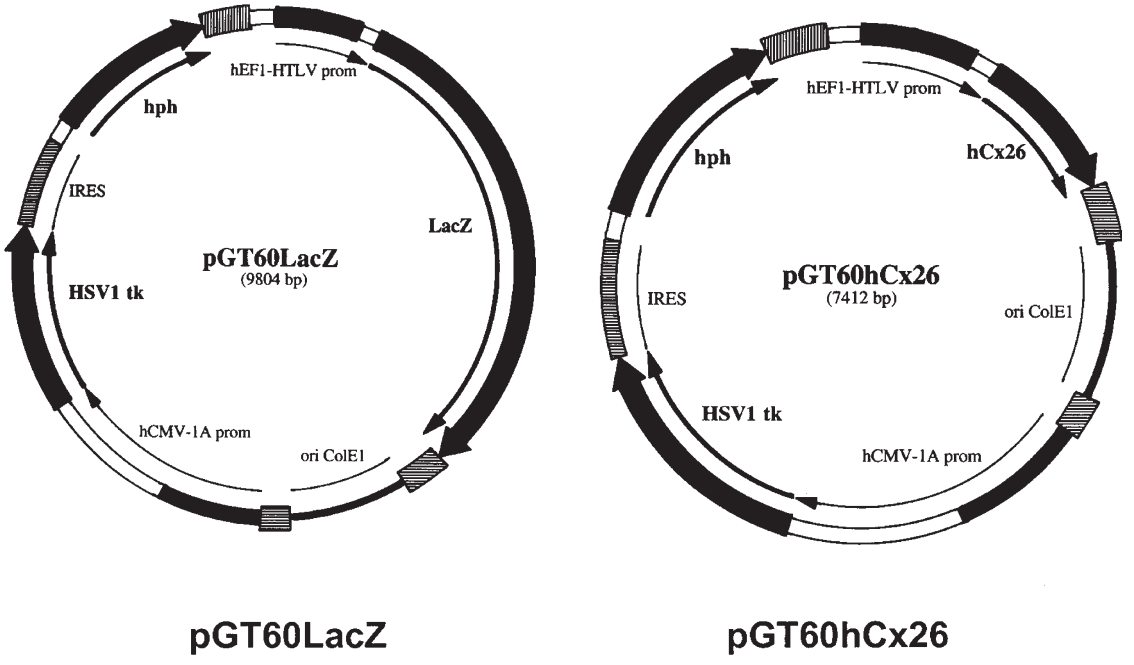

Figure 1 *Multigenic expression plasmid vectors.*

Adv/PLL/pGT60hCx26 complexes at 100 or 200 MOI, we scraped the cell monolayer and observed extensive intercellular spread of fluorescence (Figure 5b, d) compared with limited-dye transfer in UM-UC-3 and UM-UC-14 cells infected with Adv/PLL/pGT60LacZ complexes (Figure 5a, c). Phase-contrast microscopy of each cell line showed confluent monolayers and the needle tract where lucifer yellow dye was scrape-loaded. These results were reproducible. At the lowest MOI in UM-UC-3 and UM-UC-14 cells, there was less extension dye transfer (data not shown). In the cytotoxicity assays, we observed significant bystander effects in both UM-UC-3 and UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes at 50 and 100 MOI compared with cells infected at 25 and 50 MOI (Figure 2a, b). The increased bystander effect in both UM-UC-3 and UM-UC-14 cells was dose dependent and correlated with Cx 26 expression and the induction of functional gap junctions by Adv/PLL/pGT60hCx26 complexes (Figures 2a, b and 3).

In vivo bystander effect of Cx 26 transduced cells in nude mice

To test the effect of Cx 26 on enhancing the bystander effect *in vivo*, tumorigenicity was assessed in nude mice. Cells infected with either Adv/PLL/pGT60hCx26, Adv/PLL/pGT60LacZ, or Adv/PLL without plasmid DNA were injected into the dorsal flanks of nude mice. The mice were then split into groups with some beginning treatment with GCV 24 h later. Tumorigenicity was assessed 21 days after tumor cell inoculation (Table 2). UM-UC-3 cells infected with Adv/PLL/pGT60hCx26 resulted in decreased tumor volume but no change in tumor incidence in comparison to cells infected with Adv/PLL/pGT60LacZ. This represents a modest tumor suppressive effect of the CX 26 construct. Similarly, in mice treated with GCV, Adv/PLL/pGT60LacZ decreased tumor volume but not tumor incidence in comparison to Adv/PLL without plasmid DNA. This represents the effect of HSV*tk*/GCV gene therapy. On the other hand, dramatic decreases in both tumor volume and tumor incidence were found in mice treated with GCV and Adv/PLL/pGT60hCx26. These results demonstrate that induction of Cx 26 expression enhances the bystander effect *in vivo* as well as *in vitro*.

Discussion

The tools and concepts of gene therapy are being applied to the development of new treatments for human cancers.[33] One example that has been explored in a variety of *in vitro* and *in vivo* studies is HSV*tk*/GCV gene therapy.[34] This efficacy of HSV*tk*/GCV gene therapy is enhanced by the bystander effect, which helps overcome the limitations of insufficient gene transduction. At this time, the bystander effect appears to be essential for the *in vivo* success of suicide gene therapy.[27,29] *In vivo* experiments have indicated that another advantage of HSV*tk*/GCV suicide gene therapy is that it elicits an inflammatory immune response that is directed against the tumors.[35,36] The induced immunogenicity to distant HSV*tk*-negative tumor cells has been termed the distant bystander effect.[37,38]

When cells become transformed, the expression of functional gap junctions decreases.[10] Cx transfection and GJIC induction lead to a decreased rate of proliferation, increased differentiation, and reversal of the cell-transformation phenotype.[12,39] Cx 43, Cx 32 and Cx 26 knockouts result in heart malfunction, an increased incidence of liver tumors, and malfunction of the placenta, respectively.[40,41]

The tissue-specific expression of Cxs is well documented.[42] For example, Cx 32 is the major gap junction protein that has been detected in human liver and kidney. In contrast, Cx 43 is expressed in fibroblasts, osteoblasts, endothelial cells and mesenchymal tissues, and Cx

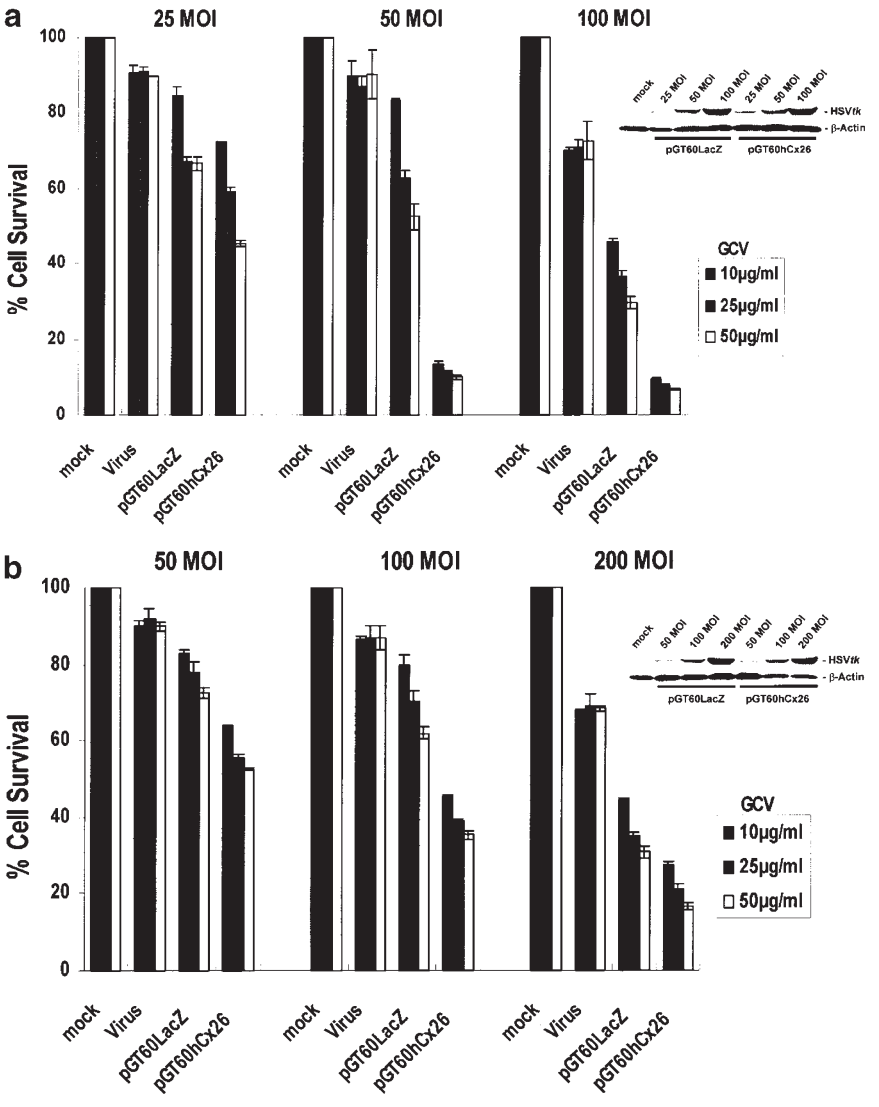

Figure 2 *The percentage of surviving cells was determined based on the number of surviving mock-infected cells. (a) Survival of UM-UC-3 cells mock-infected or infected with control Adv/PLL conjugate without plasmid (virus), Adv/PLL/pGT60LacZ complexes (pGT60LacZ), or Adv/PLL/pGT60hCx26 complexes (pGT60hCx26) at 25, 50 and 100 MOI. Cells were counted at 5 days post-infection after treatment with each of the different concentrations of GCV. (b) Survival of UM-UC-14 cells, the infection procedure was the same as that in the UM-UC-3 cells except at 50, 100 and 200 MOI. Equal transduction of HSVtk was seen in UM-UC-3 (a) and UM-UC-14 (b) cells infected with Adv/PLL/pGT60LacZ or Adv/PLL/pGT60hCx26 complexes by Western blot analysis. (c and d) Survival of UM-UC-3 (c) and UM-UC-14 (d) cells after infection with mock, control adenovirus, Adv/PLL/pGT60LacZ complexes, or Adv/PLL/pGT60hCx26 complexes but without GCV to examine the direct effect of Cx 26 as a tumor suppressor.*

26 is detected in mammary epithelial and urothelial cells.[10,42] Both Cx 26 and Cx 43 were detected in gastrointestinal tumor cell lines, but only the presence of Cx 43 correlated with the bystander effect.[21,22] The Cx 26 gene has been shown to function as a tumor suppressor gene in HeLa cells and MCF7 breast cancer cells.[13,14]

Cx 26, 32 and 43 are expressed in normal urothelium, and loss of Cx 26 is found in bladder cancer.[5] Previously, we showed that down-regulation of Cx 26 mRNA expression was associated with functional loss of GJIC in human bladder cancer, but Cx 43 expression did not correlate with GJIC in bladder cancer cells.[10] In this study, we confirmed the lack of Cx 26 expression in the human bladder cancer cell lines UM-UC-3 and UM-UC-14. After introduction of the HSV*tk* and Cx 26 genes by an Adv/PLL conjugate, we observed Cx 26 expression and a significant extension of the bystander effect in comparison with that associated with introduction of the HSV*tk* and *LacZ* genes. GCV was cytotoxic at a concentration as low as 10 μg/ml in UM-UC-3 and UM-UC-14 cells treated with Adv/PLL/pGT60hCx26 complexes but not in cells treated with Adv/PLL/pGT60LacZ complexes. In addition, Adv/PLL/pGT60hCx26 infection resulted in the formation of functional gap junctions. Although introduction of Cx 26 alone resulted in growth suppression of UM-UC-3 cells, this effect was significantly less than the cytotoxicity resulting from Cx 26 and HSV*tk*/GCV gene therapy (ie bystander effect). Our data suggest that gene therapy with Adv/PLL/pGT60Cx26 for bladder cancer is most effective as a diffuser or a dose-reducing factor in combined HSV*tk*/GCV gene therapy.

The use of an Adv/PLL conjugate with DNA is a simple, rapid, and reproducible system for testing the effect

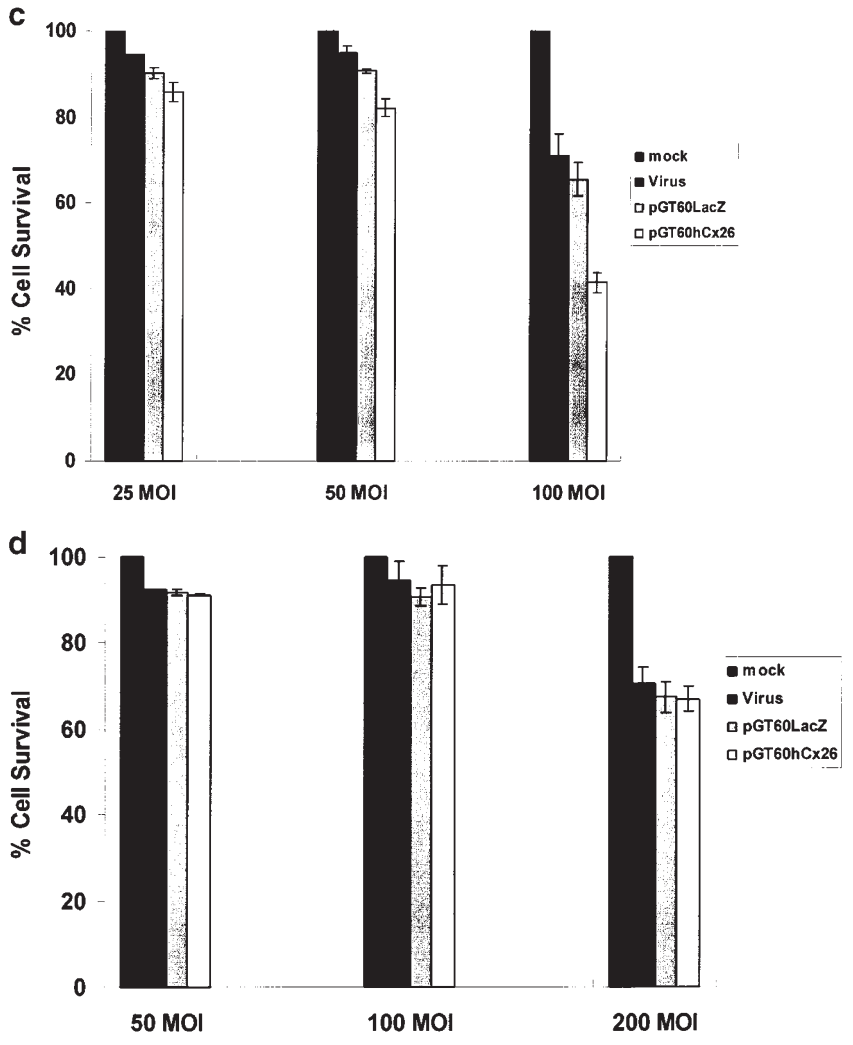

Figure 2 *Continued.*

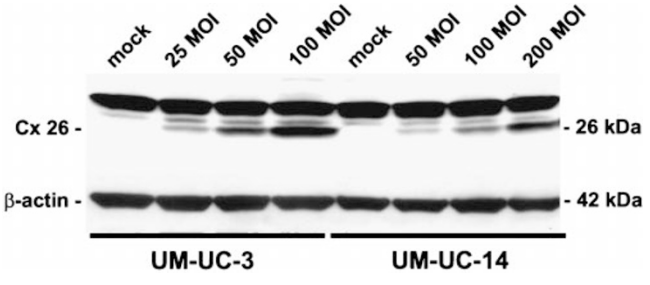

Figure 3 *Western blot analysis to detect Cx 26 expression in UM-UC-3 and UM-UC-14 bladder cancer cells infected with Adv/PLL/pGT60hCx26 complexes and medium alone (mock) at the indicated MOIs. The molecular weight of Cx 26 is 26 kDa.*

of therapeutic gene expression in cancer both *in vitro* and *in vivo*.[43–45] Furthermore, the use of a multigenic expression plasmid containing HSV*tk* and Cx 26 provided a simple system for both inducing and ensuring the expression of both genes. In the present study, the Adv/PLL conjugate was chemically modified by the covalent attachment of PLL to a replication-defective adenoviral capsid, allowing for direct interaction with DNA. This modified adenovirus can then act as a delivery vehicle for ionically attached plasmid DNA to PLL. Previously this Adv/PLL conjugate has been used to achieve high-level *p53* gene delivery in lung cancer cells *in vitro* and *in vivo*.[46] and efficient gene transfer into various cancer cell lines, including lung, cervical, colon, and esophageal cancer cell cells, as well as into fibroblasts, normal human bronchial epithelial cells, and normal human skeletal cells *in vitro*.[44,45] However, analysis of *in vitro* transduction efficiencies using X-gal (LacZ) staining revealed low efficiency compared with that of Cx 26 expression at the same MOI shown by immunofluorescence. This difference may reflect the low sensitivity of X-gal (LacZ) staining in comparison with the sensitivity of immunostaining.[47] In fact, we found significantly increased cytotoxicity mediated by the bystander effect in HSV*tk*/GCV gene therapy after introduction of the HSV*tk* and Cx 26 genes together compared with the cytotoxicity seen after introduction of the HSV*tk* gene and *LacZ* genes *in vitro* and *in vivo*. Similarly, combination therapy of HSV*tk* and Cx 43 compared with an isogenic HSV*tk* vector significantly induced the bystander killing effect in Cx-negative L929 fibrosarcoma cells *in vitro* and Cx-positive U-87 MG human glioblastoma cells *in vivo*.[48]

The recombinant adenovirus systems can efficiently deliver genes both *in vitro* and *in vivo* but require a helper

cell line and homologous recombination to generate a recombinant adenovirus. As a result, it may take several months for these labor-intensive and time-consuming techniques to generate the recombinant adenovirus vector. The Adv/PLL conjugate system provides a simple method for delivering and testing therapeutic gene expression in cells before the development of more traditional gene therapy vectors such as adenoviral or retroviral vectors. These data using an Adv/PLL conjugate indicate that restoring Cx 26 function in bladder cancer cells increases the effectiveness of HSV*tk*/GCV gene therapy.

This is the first demonstration of the bystander effect in bladder cancer. The functional status, regulation, and expression of Cx 26 should be considered in the design of HSV*tk*/GCV gene therapy for bladder cancer.

Materials and methods

Cell lines and plasmid vectors

Human bladder cancer cell lines UM-UC-3 and UM-UC-14 were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated FBS at 37°C in an atmosphere of 5% CO_2.[49]

We used the multigenic expression plasmid vectors pGT60LacZ and pGT60hCx26 (obtained from InvivoGen, San Diego, CA, USA; Figure 1). The pGT60lacZ contains the HSV*tk* gene under the control of the complete human immediate-early cytomegalovirus (hCMV) promoter-enhancer, and the β-galactosidase (*LacZ*) gene under the control of the elongation factor-1α (EF-1α)/human T cell leukemia virus (HTLV) type 1 long terminal repeat (LTR) hybrid promoter. The pGT60hCx26 contains the HSV*tk* gene and substitutes the human Cx 26 gene for the *LacZ* gene.

Adv/PLL/DNA complex formation and in vitro administration

The Adv/PLL conjugate was produced as described previously.[44–46] For the *in vitro* administration of Adv/PLL/DNA complexes, 10 μg DNA in 250 μl HBS (150 mM NaCl, 20 mM Hepes, pH 7.3) was added to 1×10^{10} modified adenoviral particles in 333 μl HBS and incubated at room temperature for 30 min. An additional 250 μl HBS was then added, and the mixture was incubated for another 30 min at room temperature. The final concentrations of the Adv/PLL/DNA complexes in 833 μl were 0.012 microgram per microliter of DNA and 1.2×10^7 virus particles per microliter of adenovirus. The cells were infected with Adv/PLL/DNA complexes according to the MOI, defined as the number of modified adenovirus particles per cell.

Uptake and expression of Adv/PLL/DNA complexes in cells

UM-UC-3 and UM-UC-14 bladder cancer cells were seeded in 35-mm six-well plates. Twenty-four hours after plating, the number of cells was determined, and the cells were infected with Adv/PLL/DNA complexes at concentrations ranging from 25 to 500 MOI or mock in the presence of culture medium containing 2% FBS for 2 h at 37°C. Forty-eight hours after infection, *in vitro* transduction efficiencies were determined by X-gal (Sigma, St Louis, MO, USA) staining.[50] Blue-staining cells were counted to determine the transduction efficiency.

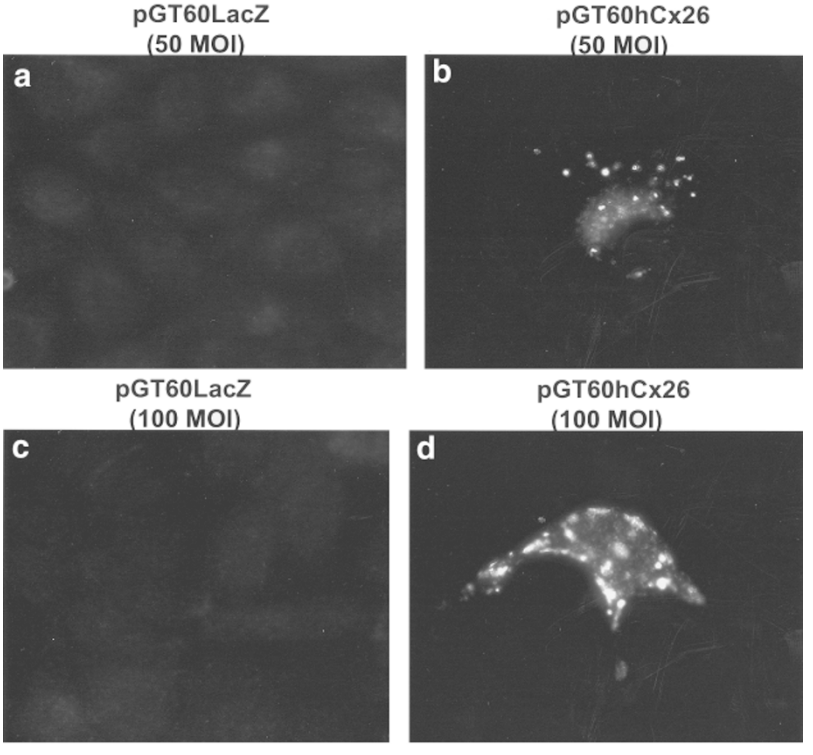

Figure 4 *Immunolocalization of Cx 26 in UM-UC-3 (b) and UM-UC-14 (d) bladder cancer cells after exogenous introduction of the Cx 26 gene by infection with Adv/PLL/pGT60hCx26 complexes compared with UM-UC-3 (a) and UM-UC-14 (c) cells infected with Adv/PLL/pGT60LacZ complexes at 50 and 100 MOIs, respectively (original magnification × 400).*

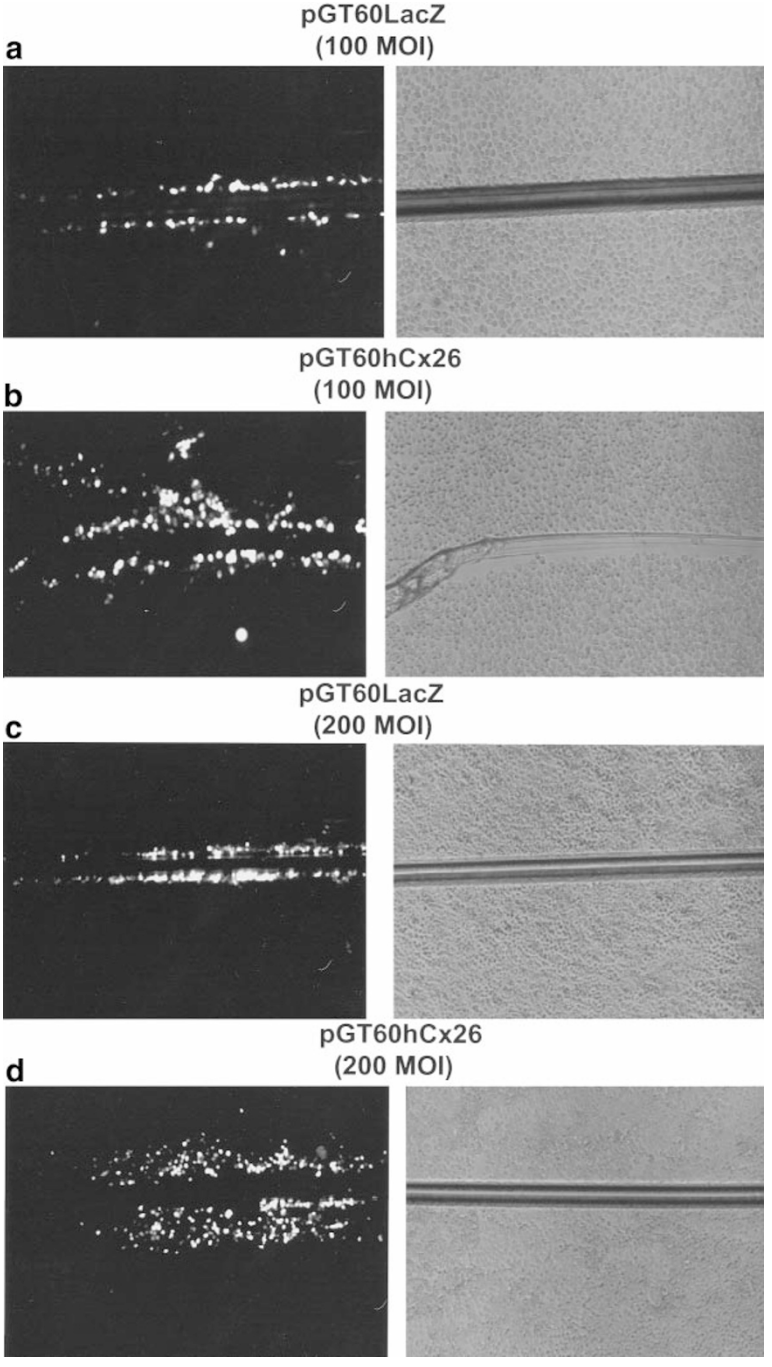

Figure 5 *GJIC-mediated dye transfer in UM-UC-3 and UM-UC-14 bladder cancer cells by the scrape-loading lucifer yellow dye transfer assay. Fluorescent and transmitted light images were taken under identical conditions for direct comparison (original magnification ×100). (a) Left, fluorescent photomicrograph of UM-UC-3 cells infected with Adv/PLL/pGTLacZ complexes. Right, phase-contrast photograph showing corresponding field of UM-UC-3 cells infected with Adv/PLL/pGTLacZ complexes. (b) Left, fluorescent photomicrograph of UM-UC-3 cells infected with Adv/PLL/pGT60hCx26 complexes showing lucifer yellow dye transfer. Right, phase-contrast photograph showing corresponding field of UM-UC-3 cells infected with Adv/PLL/pGT60hCx26 complexes. (c) Left, fluorescent photomicrograph of UM-UC-14 cells infected with Adv/PLL/pGTLacZ complexes. Right, phase-contrast photograph showing corresponding field of UM-UC-14 cells infected with Adv/PLL/pGTLacZ complexes. (d) Left, fluorescent photomicrograph of UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes showing lucifer yellow dye transfer. Right, phase-contrast photograph showing corresponding field of UM-UC-14 cells infected with Adv/PLL/pGT60hCx26 complexes.*

Cytotoxicity assay

UM-UC-3 and UM-UC-14 bladder cancer cells were plated at a density of 5×10^4 and 1×10^5 cells per well, respectively, and infected as described above with Adv/PLL conjugate without plasmid DNA (control adenovirus), Adv/PLL/pGT60LacZ complexes, or Adv/PLL/pGT60hCx26 complexes at concentrations ranging from 25 to 200 MOI. The cells were changed to selective medium containing different concentrations of GCV (10, 25 and 50 μg/ml) 24 h after infection. The

Table 2 Tumor formation in nude mice injected with UM-UC-3 cells infected *ex vivo* with Adv/PLL/pGT60hCx26, Adv/PLL/pGTLacZ or Adv/PLL complexes without plasmid DNA

Vectors + GCV	*No. of mice with tumors/ No. of mice injected*		*Mean tumor volume (mm^3) ± s.d.*	
GCV(+)				
Adv/PLL/pGT60hCx26	1/5[a]		3.8 ± 8.4[b]	$P < 0.002$
Adv/PLL/pGTLacZ	5/5	$P < 0.00003$	620.3 ± 48.2	$P < 0.002$
Adv/PLL without plasmid DNA	5/5		974.7 ± 55.2	
GCV(–)				
Adv/PLL/pGT60hCx26	3/3		824.8 ± 74.9	$P < 0.04$
Adv/PLL/pGTLacZ	3/3		900.2 ± 34.6	

[a]Number of mice with tumors is significantly different from Adv/PLLpGT60lacZ and Adv/PLL/without plasmid DNA ($P < 0.03$, Fisher's exact test).
[b]Difference in mean tumor volume treated is statistically significant as shown (unpaired Student's *t* test).

medium was replaced every 2 days. Five days after infection, the cells were trypsinized, and the number of viable cells was determined by trypan blue exclusion.

Immunofluorescence and Western blot analysis

UM-UC-3 and UM-UC-14 bladder cancer cells (5×10^4 cells) were plated in eight-well chambered culture slides (Becton Dickinson Labware, Franklin Lakes, NJ, USA) for 24 h and infected with Adv/PLL/pGT60hCx26 complexes at 50, 100 or 200 MOI. Two days after infection, the slides were washed twice with PBS and fixed in methanol for 10 min and then in acetone for 5 min. The fixed cells were blocked in 10% FBS in phosphate-buffered saline (PBS) for 30 min before incubation with monoclonal mouse anti-connexin 26 (Zymed, San Francisco, CA, USA) for 1 h. The slides were washed with PBS and incubated with goat anti-mouse IgG FITC conjugate (Sigma). The slides were mounted on coverslips with Vectashield (Vector Laboratories, Burlingame, CA, USA) and observed with a fluorescence microscope. The fluorescent images were digitized using MetaMorph version 3.6a (Universal Imaging Corp., West Chester, PA, USA).

For protein analysis, cancer cells were infected with Adv/PLL/pGT60hCx26 complexes at 25, 50, 100 or 200 MOI, as described above. Two days after infection, the cells were harvested in lysis solution containing 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 10% glycerol, 1 μM pepstatin, 1 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin. Western analysis was performed as described previously.[51] Immunoblotting was carried out using a rabbit polyclonal antibody to Cx 26 (Zymed) and a mouse monoclonal antibody to HSV*tk* (provided by Dr WC Summers, Yale University, New Haven, CT, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit or anti-mouse; Santa Cruz Biotechnology, Santa Cruz, CA, USA). A human monoclonal antibody to β-actin (Boehringer Mannheim Biochemica, Mannheim, Germany) was used as an internal control. Protein–antibody complexes were detected by an enhanced chemiluminescence system (ECL-Plus; Amersham, Piscataway, NJ, USA) on radiographic film.

Functional cell communication assay

GJIC was measured by the scrape-loading lucifer yellow dye transfer assay.[52] UM-UC-3 and UM-UC-14 bladder cancer cells (5×10^4 and 1×10^5 cells, respectively) were plated in 12-well plates for 24 h and then infected with Adv/PLL/pGT60hCx26 or Adv/PLL/pGT60LacZ complexes as described above. Two days after infection, the culture medium was removed from confluent cultures, and cells were washed twice with PBS. Monolayers were then covered with 200 μl of 0.05% lucifer yellow (Molecular Probes, Eugene, OR, USA) and scratched with a 20-gauge needle. The dye solution was removed after incubation for 5 min, and cells were washed twice with PBS. The cells were observed with a fluorescence-inverted microscope and photographed to document the degree of dye transfer.

In vivo experiments

UM-UC-3 cells were infected *in vitro* as described above with Adv/PLL conjugate without plasmid DNA (control adenovirus), Adv/PLL/pGT60LacZ complexes, or Adv/PLL/pGT60hCx26 complexes at 50 MOI. To determine the tumor growth, 4- to 6-week-old male nude mice (nu/nu; Harlan, Indianapolis, IN, USA) were used to inject 1×10^6 tumor cells subcutaneously 24 h after infection. Twenty-four hours after tumor cell inoculation, mice received i.p. injection of GCV (10 mg/kg; twice a day) for 6 days. Tumor formation was measured using a linear caliper. The tumor volume was calculated using the equation, vol (mm^3) = $A \times B^2 \times 0.5236$, where A is the largest dimension and B is the perpendicular diameter. All animal experiments were conducted under institutional guidelines established for the Animal Core Facility at MD Anderson Cancer Center and maintained by the Core Facility.

Statistical analysis

The results are presented as mean ± standard deviation (s.d.). Fisher's exact test and Student's *t* test were used with $P < 0.05$ considered statistically significant.

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