bcl-\textit{x}s Gene Therapy Induces Apoptosis of Human Mammary Tumors in Nude Mice¹

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Abstract

Bcl-x\textsubscript{s} is a dominant negative repressor of Bcl-2 and Bcl-x\textsubscript{L}, both of which inhibit apoptosis. We used a replication-deficient adenoviral vector to transiently overexpress Bcl-x\textsubscript{s} in MCF-7 human breast cancer cells, which overexpress Bcl-x\textsubscript{s}. Infection with this vector induced apoptosis in vitro. We then determined the effects of intratumoral injection of bcl-x\textsubscript{s}, adenovirus on solid MCF-7 tumors in nude mice. Tumors injected four times with the bcl-x\textsubscript{s} adenovirus showed a 50% reduction in size. Using terminal transferase-mediated dUTP-digoxigenin nick end labeling, we observed apoptotic cells at sites of bcl-x\textsubscript{s} adenoviral injection. These experiments demonstrate the feasibility of using bcl-x\textsubscript{s} gene therapy to induce apoptosis in human breast tumors.

Introduction

Overexpression of genes that inhibit programmed cell death (apoptosis) may play a role in the etiology of a variety of cancers (1), including breast cancer. Genes in these pathways, therefore, may represent novel targets for cancer therapy. The proto-oncogene bcl-2 is overexpressed in up to 70% of breast cancers (2) and may be an important negative regulator of apoptosis in these cancers. The Bcl-2 protein was first described as being overexpressed in follicular lymphomas, in which it participates in tumor formation by blocking cell death (3, 4). The ability of Bcl-2 overexpression to inhibit apoptosis has been verified in vivo using transgenic mice (5). A genetic homologue of bcl-2 named bcl-x has been cloned. Due to alternate mRNA splicing, the bcl-x gene is transcribed into long (bcl-x\textsubscript{l}) and short (bcl-x\textsubscript{s}) forms (6). The Bcl-x\textsubscript{s} protein, like Bcl-2, functions as an inhibitor of apoptosis (6–8). Overexpression of Bcl-x\textsubscript{s} has been shown to protect human neuroblastoma cells from apoptosis induced by the chemotherapeutic agents 4-hydroperoxycyclophosphamide and cisplatin (7). In contrast, the Bcl-x\textsubscript{l} protein functions as a dominant negative repressor of Bcl-2 and Bcl-x\textsubscript{s} (6, 9). We have previously reported that 4-fold overexpression of Bcl-x\textsubscript{s} in MCF-7 human breast cancer cells, which express Bcl-2 and overexpress Bcl-x\textsubscript{s}, sensitized these cells to apoptosis induced by the chemotherapeutic agents etoposide and taxol (10). The inability to produce stably transfectable clones that overexpress Bcl-x\textsubscript{s} by more than 4-fold suggests that higher levels of Bcl-x\textsubscript{s} may be lethal to cells. Therefore, we hypothesized that transient overexpression of large amounts of Bcl-x\textsubscript{s} would induce apoptosis in the absence of other insults. To test this, we used adenovirus-mediated transfer of a bcl-x\textsubscript{s} minigene to transiently overexpress high levels of Bcl-x\textsubscript{s} in MCF-7 cells. We report that this transduction caused apoptosis in vitro. In other systems, such as prostate and head and neck cancer, adenoviral-mediated transfer of the tumor suppressor genes p53 and p21 have been shown to inhibit solid tumor growth in vivo (11, 12). Based on our in vitro data, we have extended our studies to examine the effect of adenovirus-transduced bcl-x\textsubscript{s} on MCF-7 tumor growth in vivo. We report that such bcl-x\textsubscript{s} gene therapy retards the growth of solid MCF-7 tumors in nude mice by the induction of apoptosis in vivo.

Materials and Methods

Construction of bcl-x\textsubscript{s} and lacZ Adenoviruses. The bcl-x\textsubscript{s} minigene was excised from the plasmid pBSbcl-x\textsubscript{s} (provided by G. Nuñez; Ref. 6). The bacterial lacZ gene (with nuclear localization sequence) or bcl-x\textsubscript{s} minigene was inserted into the BamHI site of the pAd5RSV plasmid. This plasmid was derived by inserting the RSV\textsuperscript{L} promoter into the BglII site of the pAdBglII vector. The bcl-x\textsubscript{s} and lacZ recombinant adenoviruses were isolated by in vivo homologous recombination between the pAd5RSVbcl-x\textsubscript{s} or pAd5RSVlacZ plasmid and replication-deficient sub360 adenovirus, which has a deletion of the EIA and EIB genes, as described (9). Large preparations of the adenovirus were made by infecting 293 kidney cells and purifying crude virus by cesium chloride centrifugation. The viral titer was quantified by spectrophotometry.

Growth Studies of MCF-7 Cells. MCF-7 human breast cancer cells were cultured in MEM plus l-glutamine (Celox, Hopkins, MN) supplemented with 10% fetal bovine serum, 10 μg/ml insulin, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acid solution (Sigma Chemical Co., St. Louis, MO). For growth studies, MCF-7 cells were first plated at 10⁴ cells/well for 24 h, then treated with serum-free MEM containing the bcl-x\textsubscript{s} or lacZ adenovirus at 2–5 × 10⁴ pfu/cell for 3 h. This medium was replaced with MEM plus insulin plus 4% fetal bovine serum overnight, then full culture medium. Cell growth was assessed by cell counts using a Coulter counter (Coulter Electronics, Inc., Hialeah, FL) and by hemocytometry.

Western Blot Analysis. Protein samples were resolved by denaturing SDS-PAGE and Western blotted by standard methods. Bcl-x\textsubscript{s} and Bcl-x\textsubscript{l} were detected using a rabbit polyclonal IgG to human Bcl-x (Santa Cruz Biotechnology, Santa Cruz, CA). A goat antirabbit antibody conjugated to horseradish peroxidase and enhanced chemiluminescence (Amersham, Arlington Heights, IL) were used to visualize protein bands.

Quantitation of Apoptosis by ELISA. To quantitate the relative number of apoptotic cells in vitro, we used an ELISA (Boehringer Mannheim, Indianapolis, IN), which measures cytoplasmic DNA-histone complexes generated during apoptotic DNA fragmentation, as described previously (10).

Growth of MCF-7 Solid Tumors in Nude Mice. Six-week-old, female, athymic nude mice (CD-1, nu/nu; Charles River Breeding Laboratories, Wilmington, MA) were implanted with 0.72 mg 17β-estradiol pellets (6-week time release; Innovative Research of America, Sarasota, FL). Two days later, 6 × 10⁴ MCF-7 cells were injected s.c. in 0.1 ml 50% matrigel (Collaborative Biomedical Products, Bedford, MA) plus 50% unsupplemented MEM, as described (13). This method has yielded a 95% success rate for the formation of MCF-7 tumors in vivo.

Adenoviral-mediated Bcl-x\textsubscript{s} Overexpression in Vivo. One of two tumors per mouse was treated by direct intratumoral injection with the bcl-x\textsubscript{s} adenovirus, and the other was injected with the lacZ adenovirus, using a 0.1-ml syringe with a 26-gauge, 0.5-inch needle. A single needle puncture was made into the skin covering the tumor; then, the needle tip was moved to five tumor centers.

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different sites within the tumor, and 10 μl adenovirus preparation were injected at each site. Tumor volume was measured every 3-4 days. Linear calipers were used to measure the longest axis (a) and the width perpendicular to this axis (b). Tumor volume was calculated as: volume = a × b² × 0.4.

X-gal Staining and Detection of Apoptosis by TUNEL. Mice were sacrificed, and tumors were frozen in OCT embedding medium (Miles, Inc., Elkhart, IN) by standard methods. β-Gal activity was detected by X-gal staining on 20-μm cryosections as described (14). Internucleosomal DNA cleavage characteristic of apoptotic cells was detected in situ on 8-μm cryosections by TUNEL (ApopTag kit; Oncor, Gaithersburg, MD) as described (15).

Statistics. For ELISA data and in vitro cell counts, statistical significance was calculated by Student’s paired t test. For in vivo tumor growth experiments, overall statistical significance between control and treated groups was calculated by repeated-measures analysis using Statistical Analysis System software (SAS Institute, Inc., Cary, NC). P values are given in each figure legend.

Results

Overexpression of Bcl-xA by an Adenoviral Vector Induces Apoptosis of MCF-7 Human Breast Cancer Cells in Vitro. Expression of Bcl-x family members by MCF-7 cells was assayed by Western blotting using a rabbit polyclonal antibody that recognizes both Bcl-xL (M, 25,630) and Bcl-xS (M, 18,700). As shown in Fig. 1A, Lanes 3 and 4, control MCF-7 cells exclusively express Bcl-xL, and do not express Bcl-xS. The effects of transient overexpression of Bcl-xS on MCF-7 cells in vitro were tested by infecting these cells with an adenovirus containing a bcl-xS minigene (pAdRSV-bcl-xS). Another adenovirus carrying the bacterial gene for β-gal (pAdRSV-lacZ) was used as a control. As shown in Fig. 1A, Lanes 1 and 2, MCF-7 cells infected 3 days previously with 2 × 10⁴ pfu/cell bcl-xS adenovirus express Bcl-xS in addition to Bcl-xL.

We then determined the effect of Bcl-xS overexpression on cell viability in culture. Beginning 2 days after infection, large numbers of dead, floating cells were observed in MCF-7 cultures infected with the bcl-xS adenovirus. Fig. 1B shows that 5 days after infection with 10⁴ pfu/cell, the bcl-xS adenovirus caused a 45% greater reduction in the number of viable cells, as determined by trypan blue exclusion, than the lacZ adenovirus (P = 0.010; n = 3). To determine whether these differences in viable cell numbers were caused by apoptosis induced by the bcl-xS adenovirus, we used an ELISA. This assay quantitates cytoplasmic, histone-bound DNA derived from internucleosomal DNA fragmentation, which occurs in the nuclei of apoptotic cells. Table 1 shows that cells infected with 2 × 10⁴ pfu/cell bcl-xS adenovirus had 6-fold greater cytoplasmic DNA-histone than uninfected controls and 4.6-fold greater DNA-histone than lacZ controls (P ≤ 0.01; n = 3). These data suggest that the bcl-xS adenovirus, specifically, induces apoptosis in MCF-7 cells, accounting for the observed decrease in viability.

We then tested whether the MCF-7 cells which remained viable by trypan blue exclusion after bcl-xS infection were clonogenic. To accomplish this, we tested the ability of bcl-xS- or lacZ-infected MCF-7 cells to grow as solid, s.c. tumors in estrogen-supplemented nude mice. Three × 10⁴ pfu bcl-xS, or lacZ adenovirus were added to 3 × 10⁶ MCF-7 cells immediately prior to s.c. injection into the flank of a mouse. Fig. 1C shows that although the bcl-xS-infected cells formed a small nodule, the nodule did not grow (P = 0.0085; n = 2). The lacZ-infected cells formed larger nodules, which, after a growth delay, began to grow at a rate similar to that in uninfected controls. After 25 days, bcl-xS-infected tumors were 91% smaller than lacZ-infected controls. These results indicate that the MCF-7 cells which escaped trypan blue following bcl-xS infection were incapable of growing as a tumor in vivo, suggesting that these cells were no longer clonogenic. When titers higher than 3 × 10⁷ pfu were tested, the adenovirus vector alone was toxic to the cells, and tumors did not form.

In Vivo bcl-xS Adenoviral Treatment Retards the Growth of MCF-7 Tumors in Nude Mice. To determine whether the bcl-xS adenoviral vector would be useful for in vivo gene therapy, we extended our study to see whether in vivo bcl-xS adenoviral treatment would retard the growth of established tumors. Three-day-old MCF-7 tumors in estrogen-supplemented nude mice were treated by single injections of 7 × 10⁷ pfu bcl-xS, or lacZ adenovirus. Fig. 2A shows that tumors treated with the bcl-xS adenovirus grew at a decreased rate for 8 days after treatment, then began to grow at a rate similar to lacZ-infected tumors (P = 0.031; n = 3). Tumors treated with the lacZ adenovirus grew at a rate similar to that of untreated controls. The greatest bcl-xS-specific tumor growth inhibition was observed 8 days post cell injection.

Fig. 1. Effects of Bcl-xS expression on cell viability. A, Western blot of Bcl-x expression. MCF-7 cells were infected as described in "Materials and Methods" with 2 × 10⁴ pfu/cell bcl-xS or lacZ adenovirus, and total protein lysates (400 μg/lane) were analyzed by Western blotting using a rabbit polyclonal antibody to Bcl-x. Lanes 1 and 2, bcl-xS-infected cells; lanes 3 and 4, lacZ-infected cells. Arrows: XL, Bcl-xL (M, 25,630); XS, Bcl-xS (M, 18,700). B, effect of bcl-xS adenoviral infection on MCF-7 viability. MCF-7 cells were infected in vitro with 10⁴ pfu/cell bcl-xS or lacZ adenovirus. The number of live cells, as determined by trypan blue exclusion, was counted 5 days later (P = 0.010; bars, SE; n = 3). C, effect of bcl-xS adenoviral infection on clonogenicity of MCF-7 cells. MCF-7 cells (3 × 10⁴) were treated with 10 pfu/cell bcl-xS or lacZ adenovirus in vitro and injected s.c. into opposite flanks of an estrogen-supplemented nude mouse. Tumor volume was calculated as described in "Materials and Methods" (P = 0.0085; bars, SD; n = 2). X, no virus; Δ, lacZ; O, bcl-xS.
days after treatment, when bcl-x<sub>i</sub>-infected tumors were 38% smaller than lacZ-infected tumors. This experiment showed that in vivo bcl-x<sub>i</sub> adenoviral infection retarded tumor growth, but that single treatment was not sufficient for long-term growth inhibition.

We hypothesized that, relative to in vitro infection, in vivo infection had less effect on tumor growth because only a fraction of the cells was infected; this hypothesis was subsequently confirmed (see Fig. 3A). Therefore, we tested whether multiple in vivo infections with the bcl-x<sub>i</sub> adenovirus would cause greater or sustained retardation of tumor growth. Three-day-old MCF-7 tumors were treated with 7 x 10⁷ pfu bcl-x<sub>i</sub> or lacZ adenovirus every 4 days for five treatments. Fig. 2B shows that throughout the duration of treatment, the bcl-x<sub>i</sub>-infected tumors grew at a slower rate than the lacZ-infected tumors (P = 0.0032; n = 4). The greatest bcl-x<sub>i</sub>-specific retardation of growth was observed following four treatments (13 days after the first treatment), when bcl-x<sub>i</sub>-infected tumors were 52% smaller than lacZ-infected tumors. Thus, multiple treatments with the bcl-x<sub>i</sub> adenovirus caused greater and sustained tumor growth inhibition relative to single treatment.

**Inhibition of MCF-7 Tumor Growth in Vivo by bcl-x<sub>i</sub>, Adenoviral Infection Is Due to Induction of Apoptosis.** To determine the efficacy of our direct intratumoral approach to adenoviral-mediated gene therapy, we estimated the number of cells in an infected tumor by apoptosis, consistent with the inhibition of the overall tumor growth rate caused by this treatment.

Normal mammary epithelial cells are capable of undergoing apoptosis during involution following lactation (16). Inhibitors of this ability to undergo apoptosis, such as Bcl-2 and Bcl-x<sub>L</sub>, may play an important role in the development of breast cancer. In this communication, we report that MCF-7 human breast cancer cells overexpress Bcl-x<sub>L</sub> but do not express Bcl-x<sub>i</sub>. Bcl-x<sub>i</sub> is a dominant negative repressor of Bcl-2 and Bcl-x<sub>L</sub> and is, therefore, a putative inducer of apoptosis in cancer cells which are resistant to apoptosis due to overexpression of Bcl-2 and/or Bcl-x<sub>L</sub> (6, 9). We report that adenovirus-mediated overexpression of Bcl-x<sub>i</sub> induces apoptosis of MCF-7 human breast cancer cells in vitro. In addition, we report that adenovirus-mediated bcl-x<sub>i</sub> gene therapy can retard MCF-7 tumor growth in a nude mouse model and present evidence that this growth inhibition is due to induction of apoptosis. The bcl-x<sub>i</sub> adenovirus, which is replication deficient, caused no obvious systemic or local toxicity, such as skin necrosis, when delivered locally.

**Table 1. Quantitation of apoptosis by ELISA**

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<th>Sample</th>
<th>Mean ± SE units of cytoplasmic DNA-histone</th>
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<tr>
<td>Uninfected MCF-7</td>
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<td>bcl-x&lt;sub&gt;i&lt;/sub&gt;-infected MCF-7</td>
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<td>lacZ-infected MCF-7</td>
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**Discussion**

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Fig. 2. bcl-x<sub>i</sub> adenoviral effects on tumor growth in vivo. A, tumor growth following single injection. Three days after s.c. injection of 6 x 10⁷ pfu MCF-7 cells, tumors were injected with 7 x 10⁷ pfu bcl-x<sub>i</sub> or lacZ adenovirus (P = 0.031; bars, SE; n = 3). B, tumor growth following multiple injections. Tumors were treated five times, once every 4 days (P = 0.0032; bars, SE; n = 4). Arrows, treatment. △, LacZ; ○, bcl-x<sub>i</sub>.
In vivo bcl-x, adenoviral treatment reduced the size of the solid MCF-7 tumors by a greater amount than would be predicted from the percentage of cells that were infected as determined by lacZ expression. Single in vivo adenoviral treatment resulted in bcl-x, -infected tumors that were 38% smaller than lacZ-infected controls. However, X-gal staining showed that a single adenoviral treatment infected only about 15% of the cells in each tumor. This raises the possibility that bcl-x, adenoviral infection kills additional cells via a bystander effect, in which uninfected cells surrounding an infected cell also are killed. The bystander effect has been shown to induce apoptosis of unmodified tumor cells adjacent to cells transduced with the herpes simplex virus thymidine kinase gene and treated with ganciclovir (17). Such bystander killing was associated with the transfer of apoptotic vesicles from transduced, dying cells to adjacent untransduced cells (17). We are currently investigating whether Bcl-x, -induced apoptosis may cause a similar bystander effect.

The in vitro ELISA data and in situ TUNEL assays presented here suggest that a significant fraction of the cell killing caused by the bcl-x, adenovirus is due to apoptosis. It is not yet clear whether this apoptosis is solely the result of Bcl-x, overexpression, or whether the adenoviral vector presents an insult to the cell which contributes to the induction of apoptosis. However, the bcl-x, adenovirus caused significantly greater apoptosis than the same titer of the lacZ adenovirus, providing evidence that Bcl-x, overexpression itself contributes to apoptosis and showing the importance of blocking inhibitors of cell death, such as Bcl-2 and Bcl-x,.
viral-mediated gene transfer of bcl-x<sub>o</sub>, which causes transient overexpression, is sufficient to induce apoptosis.

A possibility for improving the efficacy of bcl-x<sub>o</sub> gene therapy may come from a combination of this approach with chemotherapy. We have previously reported that stable transfection of bcl-x<sub>o</sub> sensitized MCF-7 cells to apoptosis induced by the chemotherapeutic agents etoposide and taxol in vitro (10). By blocking inhibitors of apoptosis, bcl-x<sub>o</sub> gene therapy may lower the apoptotic threshold of cancer cells to other agents that cause cell death.

Bcl-2 and Bcl-x<sub>L</sub>, the functional targets of Bcl-x<sub>o</sub>, seem to inhibit apoptosis through a common pathway (18). Bcl-2 has been shown to be overexpressed in up to 70% of breast cancers (2) and, therefore, may play an important role in the development of these cancers. Although such data have not yet been reported for Bcl-x<sub>L</sub>, we have found Bcl-x<sub>L</sub> to be overexpressed in a significant percentage of primary cell lines and tissues derived from breast cancers (data not shown). These data suggest that Bcl-2 and Bcl-x<sub>L</sub> may be important targets for novel breast cancer treatments, such as bcl-x<sub>L</sub> adenoviral gene therapy.

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References

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