Advances in Brief

Antitumor Effect of Adenovirus-mediated *Bax* Gene Transfer on *p53*-sensitive and *p53*-resistant Cancer Lines[1]

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Abstract

Antitumor effects of the proapoptotic *Bax* gene have been evaluated *in vitro* and *in vivo* by a binary adenovirus system expressing the human *Bax* gene. Overexpression of the *Bax* gene in cultured cell lines from human lung carcinoma results in caspase activation, apoptosis induction, and cell growth suppression. Intratumoral injection of adenovirus vector expressing the *Bax* gene suppressed growth of human lung cancer xenografts established in nude mice. Histological examination of tumors from mice treated with the *Bax* gene demonstrated high levels of *Bax* expression and extensive apoptosis in tumors. In comparison with the treatment by an adenoviral vector expressing human p53, the *Bax* gene can effectively suppress tumor growth in both *p53*-sensitive and *p53*-resistant human lung carcinoma cell lines. Toxicity was not detected in liver and other systems in animals treated intralesionally with the *Bax* gene. Therefore, our results suggest that the *Bax* gene may be useful in cancer treatment.

Introduction

Evidence that antitumor therapies function by inducing apoptosis is revealing the crucial role apoptosis plays in tumorigenesis and antitumor therapy. Because cells have varying susceptibility to apoptosis induction, chemotherapy or radiation therapy may induce apoptosis in tumor cells and merely arrest the cell cycle of their normal counterparts, thereby opening a therapeutic window (1). Correspondingly, insensitivity to apoptosis induction may be a major mode of resistance to antitumor therapy. Apoptosis also directly regulates tumorigenesis. For example, *p53*, the abnormalities of which have been detected most frequently in human cancers, modulates apoptosis by regulating the expression of the *Bcl-2* and *Bax* genes (2).

The widely expressed *Bax* gene is one of the well-characterized proapoptotic genes, and its overexpression leads to apoptosis in a wide variety of cells, with or without other additional stimuli (3). The *Bax* gene also plays a crucial role in development as demonstrated by Knudson *et al.* (4), who reported hyperplasia in thymocytes and B cells and resistance to certain apoptotic stimuli in *Bax*-knockout mice. Results of other studies suggest that *Bax* mutations, such as that at codon 169, decrease the proapoptotic activity of *Bax* and play an important role in the course of carcinogenesis in the stomach, colorectum, endometrium, and hematological tissues in humans (5). Research has also shown that the *Bax* gene promoter contains *p53*-

binding sites and that expression of the *Bax* gene is up-regulated by *p53* (6). These findings suggest that the *Bax* gene is a component of the *p53*-mediated apoptotic response and acts as a tumor suppressor. The importance of *Bax* gene expression in the clinical outcome of cancer patients has also been recognized. Reduced expression of *Bax* is associated with poor response rates to combination chemotherapy and shorter survival in women with metastatic breast cancer (7). On the other hand, overexpression of *Bax* enhances chemotherapy and radiation therapy of cancers and improves the clinical outcome (8, 9). Recently, Strobel *et al.* (10) showed that *Bax* enhances intracellular accumulation of chemotherapeutics such as paclitaxel. Thus, the *Bax* gene may serve as a good candidate for cancer gene therapy, not only because it may kill cancer cells directly, but also because it may potentially increase the sensitivity of other antitumor treatments.

We have recently developed a binary adenoviral vector system with regulatory components of the *GAL4* gene that has enabled us to overcome the difficulties in constructing adenoviral vectors expressing high levels of the strong apoptotic *Bax* gene (11). This system involves an adenoviral vector containing a human bax cDNA driven by a synthetic promoter consisting of five GAL4-binding sites and a TATA box (Ad/GT-Bax). This vector expresses a minimal background level of bax protein in cultured mammalian cells, thus preventing apoptosis of packaging cells; however, expression of the *Bax* gene can be induced substantially *in vitro* and *in vivo* by transferring it into target cells along with an adenoviral vector expressing the transactivator, fusion protein GAL4/VP16 (Ad/PGK-GV16). Morphology studies have shown that overexpression of the *Bax* gene delivered with this binary adenoviral vector induces apoptosis (11). Here, we evaluate the antitumor activities of the *Bax* gene *in vitro* and *in vivo* in human lung cancer cell lines H1299 and A549. We also examined the toxic effects of intratumoral *Bax* gene delivery and compared antitumor effects of the *Bax* gene delivered by the binary vector system with that of the *p53* delivered by a single vector system. Our results showed that the *Bax* gene can effectively suppress tumor growth in both *p53*-sensitive and *p53*-resistant cancer lines.

Materials and Methods

Cell Lines and Adenoviruses. Non-small cell lung cancer cells A549 and H1299 were grown as monolayers in HAM/F12 and RPMI 1640 media, respectively, and supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. The adenoviral vectors used in this study are the following: recombinant adenoviruses regulated by the GT[3] minimal synthetic promoter (11) and containing human *Bax* gene cDNA (Ad/GT-Bax) or *Escherichia coli* β-galactosidase gene (Ad/GT-LacZ); the GV16 transactivating protein for the GT promoter under the control of the PGK promoter (Ad/PGK-GV16; Ref.

[3] The abbreviations used are: GT, GAL4/TATA; GV16, GAL4VP16 fusion protein; PGK, 3-phosphoglycerate kinase; CMV, cytomegalovirus; GFP, green fluorescent protein; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphenyl)-(2*H*)-tetrazolium-5-carboxanilide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling; AST, aspartate transaminase; ALT, alanine transaminase.

11); human wild-type *p53* gene regulated by the immediate-early CMV promoter-enhancer (Ad/CMV-p53; Ref. 12); or an E1-deleted empty vector (AdE1$^-$; Ref. 12). All viruses were propagated in package 293 cells, purified twice by ultracentrifugation in a cesium chloride gradient, and subjected to dialysis. The titer for each virus vector was determined by the absorbency of the dissociated virus at $A_{260 nm}$ and by plaque assay (13). Titers for subsequent experiments were particles/ml determined by $A_{260 nm}$. Particles:plaque ratios were usually between 30:1 and 100:1. All viral preparations were free of E1$^+$ adenovirus contamination, determined by PCR, and free of endotoxin contamination, determined by assays with a third-generation pyrogen testing kit from BioWhittaker, Inc. (Walkersville, MD).

***In Vitro* Gene Transfer.** H1299 and A549 cells were plated 1 day before being infected with adenovirus vectors at a total multiplicity of infection of 900 and 1500 viral particles/cell, respectively. Transgene-expressing vector Ad/GT-Bax or Ad/GT-LacZ was combined with the Ad/PGK-GV16 induction vector at a 2:1 ratio, which was shown by a preliminary experiment to be the optimal ratio for inducing transgene expression. Cellular proteins were analyzed 48 h after infection by Western blotting, as described previously (11). Cell viability was determined 24, 48, and 72 h after infection by colorimetric assay with tetrazolium dye XTT (14) using the Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. The experiments were performed at least twice for each cell line. The percentages of apoptotic cells were determined by flow cytometry. Briefly, both adherent and floating cells were harvested at 24 and 48 h after infection with viral vectors and then fixed in 70% ethanol. Cells were stained either with propidium iodide for DNA contents or stained by TUNEL for DNA damage as described previously (15). Apoptotic cells were quantified by flow cytometric analysis performed at the Flow Cytometry Core Laboratory at our Institution.

Animal Experiments. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication number 85-23) and the institutional guidelines of The University of Texas M. D. Anderson Cancer Center. Human lung cancer xenografts were established in nude mice, 6–8 weeks of age (Harlan Sprague Dawley, Inc., Indianapolis, IN) through s.c. inoculation of 1×10^6 H1299 or A549 cells into the dorsal flank of each mouse. Tumors were measured two to three times a week, and tumor volume was calculated as $a \times b^2 \times 0.5$, where a and b were large and small diameters, respectively. When tumors had reached a diameter of about 0.3–0.5 cm, each mouse was given three doses of intratumoral injection of 100 μl of 9×10^{10} particles of Ad/E1$^-$, Ad/CMV-p53, or Ad/GT-Bax or Ad/GT-LacZ mixed at a 2:1 ratio with Ad/PGK-GV16. Animals were sacrificed (mandatory) when tumors reached a diameter of ~1.5 cm.

Histochemical Study. Tissue or tumor sectioning and staining with H&E were performed in the Histology Laboratory in the Department of Veterinary Medicine and Surgery at M. D. Anderson Cancer Center. For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining, 8-μm frozen sections were fixed with 0.5% glutaraldehyde for 15 min at 4°C before being stained with a solution containing 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside at 37°C overnight. The next day, sections were counterstained with Nuclear Fast Red (Sigma Chemical Co., St. Louis, MO). For immunohistochemical analysis of the Bax protein, tumors were fixed in 10% formalin, embedded in paraffin, and then cut into 4-μm sections. To retrieve antigens, the sections were baked, deparaffinized, and heated in citrate buffer (10 mM citric acid, pH 6.0) in a steamer. After endogenous peroxidase was inactivated with 10-min exposure to 1.5% H_2O_2/methanol, the sections were incubated with blocking serum (goat serum/PBS) at room temperature for 30 min (to block nonspecific binding), rabbit anti-bax polyclonal antibody (N-20; Santa Cruz Biotechnology; 1:400 dilution) for 1 h, and biotinylated goat antirabbit IgG antibody for 30 min. The specific binding were visualized with an avidin-biotin-peroxidase reagent (Vector Laboratories, Inc, Burlingame, CA) and its substrate diaminobenzidine tetrachloride (Sigma) and by counter staining with Mayer's Hematoxylin.

Analysis of Serum AST and ALT. Blood was drawn from the tail vein of mice 5 and 15 days after the last injection of adenovirus. The levels of serum AST and ALT were measured as described (12).

Statistical Analysis. Differences among the treatment groups were assessed by ANOVA using Statistical software (StatSoft, Tulsa, OK). For the experiments of tumor growth *in vivo*, ANOVA with repeated measurement module was used. $P \leq 0.05$ was considered significant.

Results

Apoptosis Profiles after Overexpression of the *Bax* Gene. We have demonstrated recently that a binary adenoviral vector system with *GAL4* gene regulatory components can efficiently induce overexpression of the *Bax* gene *in vitro* and *in vivo*, and overexpression of the *Bax* gene elicits apoptosis (11). To quantify the antitumor effects of the *Bax*-expressing vectors, TUNEL-positive populations or sub-G_1 fractions (Fig. 1A) were determined by flow cytometry at 24 and 48 h, respectively, after the treatments. The percentage of apoptotic cells determined by TUNEL assay or sub-G_1 analysis was similar in each treatment group (data not shown). Although treatment with Ad/GT-LacZ plus Ad/PGK-GV16 or Ad/GT-Bax plus Ad/CMV-GFP resulted in only background levels of apoptotic cells as that of mock infection, treatment with Ad/GT-Bax plus Ad/PGK-GV16 markedly increased the apoptotic cells in both H1299 and A549 cells (37–50%; Fig. 1).

To compare the antitumor effect of the *Bax* gene with that of the tumor suppressor gene *p53*, cell viability was determined by XTT assay at 24, 48, and 72 h after infection of Ad/CMV-p53, Ad/GT-Bax plus Ad/PGK-GV16, or Ad/GT-LacZ plus Ad/PGK-GV16. PBS was used for mock control. The *p53* gene is homozygously deleted in H1299 cells but is wild type in A549 cells (Fig. 1B). Our previous study has showed that H1299 is more sensitive to Ad/CMV-p53 treatment than A549 cells, a finding consistent with the report by others that tumor lines with wild-type p53 are relatively resistant to the treatment by *p53* gene (16). H1299 cells infected with Ad/CMV-p53 or Ad/GT-Bax plus Ad/PGK-GV16 showed a similar level of cell killing, whereas only A549 cells infected with Ad/GT-Bax plus Ad/PGK-GV16 showed massive cell death (Fig. 2), suggesting that the antitumor effect of the *Bax* gene is not dependent on *p53* status and that the *Bax* gene can be used to treat cancers resistant to *p53*.

Induction of *Bax* Gene Expression and Apoptosis in Established Tumors in Mice. To investigate whether the *Bax* overexpression and cell death can be similarly induced in tumors by adenovirus-mediated gene cotransfer, we established human lung cancer xenografts derived from H1299 and A549 cells in nude mice. Intratumoral administration of vectors were performed when tumors had reached a diameter of about 0.3–0.5 cm. Two animals from each treatment group were euthanized 2 days after three sequential treatments, and the tumors were harvested for histochemical examination. In comparison with treatments using PBS or AdE1$^-$, overexpression of the *Bax* gene was detected in all tumors treated with Ad/GT-*Bax* plus Ad/PGK-GV16 but not in tumors treated with Ad/GT-LacZ plus Ad/PGK-GV16, or Ad/CMV-p53 (Fig. 2A). Results of *in situ* TUNEL assay showed a marked increase in apoptosis within H1299 tumors treated with Ad/GT-Bax plus Ad/PGK-GV16 or Ad/CMV-p53 compared with apoptosis in H1299 cells treated with the other adenoviral vectors. In tumors derived from A549 cells, however, massive apoptosis was observed only in all of the tumors treated with Ad/GT-Bax plus Ad/PGK-GV16. A549 tumors treated with other vectors, including Ad/CMV-p53, showed only background levels of apoptosis (Fig. 2B). This finding is consistent with results of the *in vitro* study that H1299 is sensitive to both *p53* and *Bax* whereas A549 is sensitive only to *Bax*.

Suppression of Growth of Established Tumors by the *Bax* Gene. To further assess the antitumor activities of the *Bax* gene, 8–10 animals/group in the above-mentioned experiments were monitored for tumor size changes after the treatment. In tumors derived from H1299 cells, treatments with the *Bax* gene and the *p53* gene significantly suppressed tumor growth when compared with other controls ($P \leq 0.001$; Fig. 3). In tumors derived from A549 cells, only the treatment with the *Bax* gene significantly suppressed tumor growth when compared with other controls ($P = 0.009$). Tumors treated with
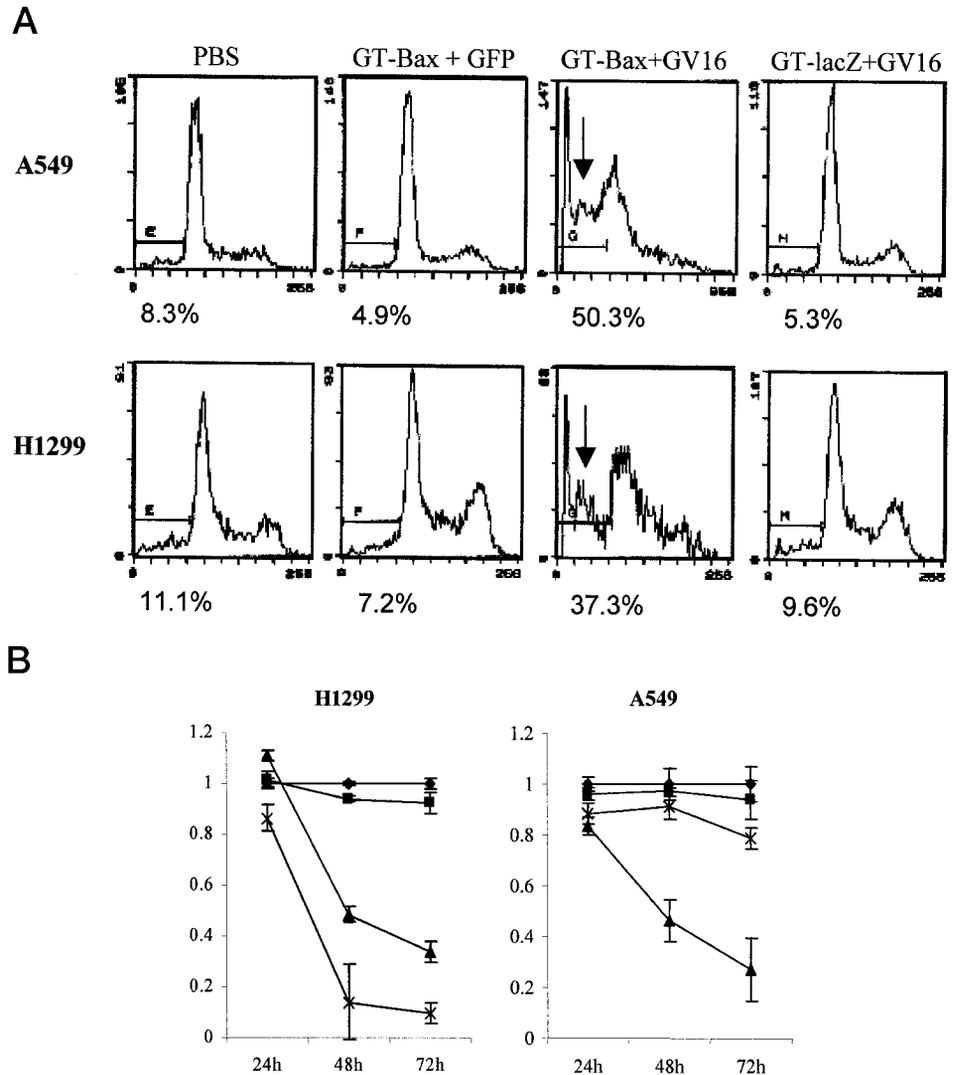
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Fig. 1. *In vitro* assessment of the antitumor effect of the *Bax* gene. *Left,* cell lines; *top,* treatments. *A,* flow cytometry for apoptotic cells (cells in the sub-G_1 phase). *B,* cell viability determined by colorimetric assay with XTT after infection. Viability value was expressed relative to that of cells infected with PBS, which was arbitrarily referred to as 1. Values for Ad/GT-Bax plus Ad/PGK-GV16 (▲) or Ad/CMV-p53 (X) were significantly different from those for PBS (♦) and Ad/GT-LacZ plus Ad/PGK-GV16 (■) groups in H1299 ($P \leq 0.01$), whereas in A549 cells, only the treatment with Ad/GT-Bax plus Ad/PGK-GV16 significantly differed from that in other groups ($P < 0.01$). Values are the means of one of two similar quadruplicate studies for each group; *bars,* SD.

Ad/CMV-p53, however, were only marginally suppressed when compared with that treated by *LacZ* gene or AdE1⁻ ($P \geq 0.15$).

Minimal Toxicity after Intratumoral Delivery of the *Bax* Gene. Using the binary adenoviral vector system, we have found previously that overexpression of *Bax* induced a massive apoptosis in liver 24 h after i.v. infusion. To evaluate the *Bax*-related toxicity after intratumoral gene delivery with the same vector system, histopathological changes in liver, spleen, kidney, adrenal grand, heart, and lung were examined for the two mice of each experimental group that were euthanized 2 days after three sequential intratumoral vector administrations as described above. No lesions were found in liver, kidney, adrenal grand, heart, and lung in all animals examined. However, extensive hyperplasia of myeloid cells and histocytes and lymphoid cells were found in spleens of the animals treated with adenoviral vectors, regardless of whether empty vector, or vectors expressing *Bax* or LacZ (data not shown), suggesting that the response in spleen was vector related rather than *Bax* related.

To further document the toxicity by the *Bax* gene treatment, blood was collected from animals 5 and 15 days after the last of the three sequential intratumoral treatments. Serum levels of liver enzymes, ALT and AST, were determined. At the both time points, all animals showed normal serum levels of the liver enzymes examined. No significant difference was found among groups (Fig. 4). Together, these results indicated that intratumoral *Bax* gene delivery is a safe and well-tolerated approach for cancer therapy.

Discussion

The antitumor effects of the *Bax* gene have been assessed recently by two different groups with different approaches. Using naked DNA transfer, Coll *et al.* (17) have showed that transfecting the *Bax* gene to two cultured bronchioloalveolar carcinoma cell lines killed 70–90% of the transfected cells, whereas p53 killed only 40% of them. Nevertheless, *Bax* and p53 shared a similar antitumor activity *in vivo* (17). However, the authors also showed that limited antitumor activity was observed in a poorly transfectable tumor line, suggesting that efficient delivery of the transgene to the target cells is an important factor in assessing the therapeutic value of a gene.

Because of their high transduction efficiencies in a variety of tissues, adenoviral vectors are widely used for *in vivo* gene delivery in gene therapy (18), and several are under clinical investigation as cancer treatment. Thus, an adenoviral vector constitutively expressing the *Bax* gene will facilitate the evaluation of its antitumor activities in a variety of cancer lines. However, constructing such a vector has been difficult, presumably because of the toxic effect of the transgene product on the package cell line 293. More recently, Tai *et al.* (19) constructed an adenoviral vector expressing the *Bax* gene under the control of a relatively tumor-specific promoter derived for the *DF3* (*MUC1*) gene. Overexpression of the *Bax* gene and cytotoxicity were observed in *DF3*-positive ovarian cancer cells. The authors showed that i.p. administration of the vector 2 and 3 days after the inoculation
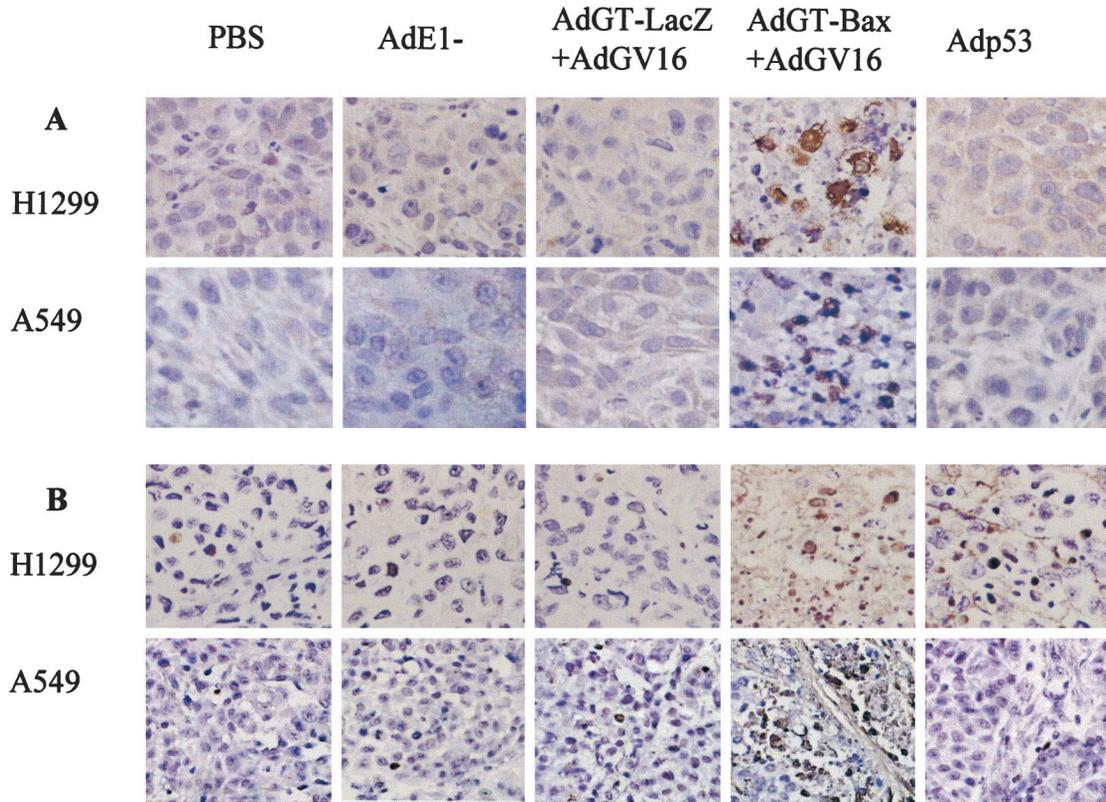
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Fig. 2. *Bax* gene expression (*A*) and *in situ* TUNEL staining for apoptosis (*B*) in established tumors.

of *DF3*-positive tumor cells significantly reduced numbers of tumor nodules, suggesting that overexpression of the *Bax* gene elicits antitumor activity.

Here we used a binary adenoviral vector system to assess the antitumor effects of the *Bax* gene *in vitro* and *in vivo* in lung cancer cell lines H1299 and A549. Because the PGK promoter is ubiquitously active in mammalian cells, this system may also be useful for testing the antitumor activities of the *Bax* gene in a variety of tumor models. The results we obtained demonstrated that the *Bax* gene can effectively induce apoptosis and suppress tumor growth both *in vitro* and *in vivo*. Furthermore, our results demonstrated that the antitumor effects of the *Bax* gene are independent of the p53 status in cells, and the *Bax* gene can effectively kill both p53-sensitive and p53-resistant tumors *in vitro* and *in vivo*. Although we have only tested three sequential intratumoral injections in this study, other administration schedules or subsequent challenges with the *Bax*- expressing vectors may theoretically improve the therapeutic effects further by minimiz-

ing untransduced tumor cells, unless clonal resistance to bax-mediated apoptosis occurs. On the basis of the evidence that overexpression of *Bax* enhances intracellular accumulation of chemotherapeutics (10) and improves the clinical outcome of chemotherapy and radiation therapy of cancers (8, 9), the combination of the *Bax* gene therapy with other therapeutic agents may also improve the therapeutic effects.

As a strong proapoptotic gene, overexpression of the *Bax* gene may induce apoptosis in normal cells as well. In fact, we have demonstrated previously i.v. infusion of the *Bax*-expressing adenoviral vector induced a rapid and massive apoptosis in hepatocytes (11). This raises a safety issue if the *Bax* gene is used as a therapeutic agent. Thus, targeted expression of the *Bax* gene is highly desirable. Nevertheless, intratumoral delivery of the *Bax* gene did not cause detectable toxicity in animals, suggesting that intratumoral delivery of the *Bax* gene appears safe for use as treatment for primary tumors. Unresectable primary cancers in brain, lung, pancreas, head and neck,

Fig. 3. Suppression of tumor growth by adenovirus-mediated gene transfer. s.c. tumors derived from H1299 (*A*) and A549 (*B*) cells treated with PBS (◆), Ad/E1⁻ (■), Ad/GT-LacZ plus Ad/PGK-GV16 (▲), Ad/GT-Bax plus Ad/PGK-GV16 (●), or Ad/CMV-p53 (X) are shown. Tumor volume was monitored over time (days) after inoculation of tumor cells. *Arrow,* time point where treatment was given. Values represent the means of at least eight mice per group; *bars,* SE. Treatments with the *Bax* and *p53* gene differ significantly from other control groups ($P \leq 0.001$) in the H1299 tumor model, whereas only the treatment of the *Bax* gene differs significantly from controls in the A549 tumor model ($P = 0.009$).
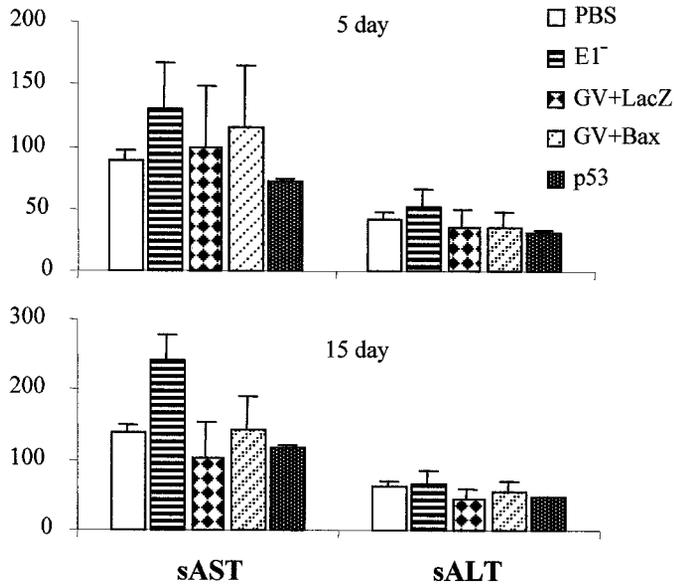
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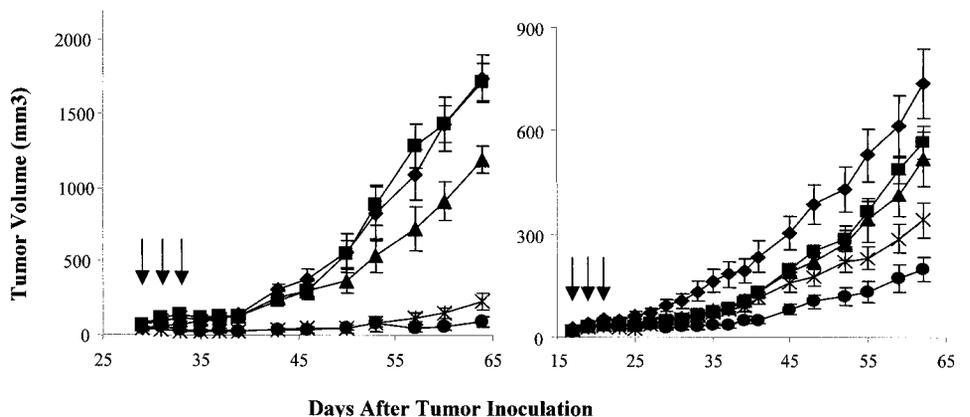
Fig. 4. Serum levels of AST and ALT 5 and 15 days in mice whose treatment results are shown in Fig. 3. All values are within the normal range. Values represent the means of three animals per group; *bars,* SD.

and others remain the major cause of morbidity and mortality in cancer patients and are one of the most formidable problems in clinics. In lung cancer, for example, despite the use of chemotherapy and radiation therapy in combined modality protocols, local control rates are <20% (20). However, treatment of metastatic tumors by systemic gene delivery remains challenging. Development in vector targeting and targeted transgene expression, used alone or combined with other approaches, may be helpful for such an approach.

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