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Bax-Induction Gene Therapy of Pancreatic Cancer

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Background. **Bax is a strong pro-apoptotic gene that induces programmed cell death when expressed. Human telomerase reverse transcriptase (hTERT) is the catalytic subunit for telomerase, an enzyme found to be active in more than 85% of human cancers. Recently, a binary adenoviral system (Ad/GT-*Bax* + Ad/hTERT-GV16) was constructed using the hTERT promoter to induce *Bax* gene expression in tumor cells.**

Methods. **To test whether human pancreatic tumor cells would respond to this system of *Bax*-induced apoptosis, we compared the effects of *Bax* gene induction with that of *LacZ* gene induction using the same binary system.**

Results. **Lysates of the human pancreatic cell lines PANC-28, MIA PaCa-2, and BxPC-3 showed significantly elevated levels of human telomerase using the PCR-based TRAP assay. As early as 24 h after treatment with *Bax*-induction gene therapy, growth inhibition was observed. Overexpression of the *Bax* protein was confirmed by Western blotting. Extensive apoptosis on FACS analysis at 48 h was seen after *Bax* induction. In addition, cytosolic cytochrome *c* levels increased compared to mitochondrial levels after *Bax* induction. Levels of caspase-3, a key downstream enzyme involved in apoptosis, also increased significantly compared to controls after treatment. None of these effects were seen with *LacZ*.**

Conclusion. **Our results suggest that the binary adenoviral vector system, Ad/GT-*Bax* + Ad/hTERT-GV16, induces high levels of *Bax* expression that induce apoptosis in human pancreatic cancer cells.** © 2002 Elsevier Science (USA)

Key Words: **pancreatic cancer; gene therapy; *Bax*; telomerase; cytochrome *c*; caspase-3.**

INTRODUCTION

Pancreatic cancer is the fourth leading cause of adult cancer deaths in the United States [1]. Approximately 29,000 new cases of adenocarcinoma of the pancreas are diagnosed each year in the United States and approximately 28,900 patients die of this malignancy [1]. Furthermore, only 1 to 4% of all diagnosed pancreatic patients can expect to survive 5 years [2]. Therefore, given the aggressive nature of pancreatic cancer, attention has turned to the use of therapeutic approaches that target the disease at the molecular level [3, 4].

Bax is a gene that is part of the Bcl-2 family of genes that relay pro-survival or pro-apoptotic signals to the cell. *Bax* activates the caspase pathway and induces release of apoptotic molecules such as cytochrome *c* from the mitochondria to the cytosol. Therefore, when high levels of *Bax* are expressed, the cell is induced to commit cell suicide, leading to cell death. Overexpression of *Bax* has been shown to induce apoptosis in a wide variety of cell lines including prostate, colon, cervical, and ovarian cancers [5–9].

Recently, a tumor-selective binary adenoviral vector system has been shown to be effective in halting the growth of cancer cells by inducing apoptosis via overexpression of *Bax* under the control of the telomerase promoter [10]. The first adenoviral vector (Ad/GT-*Bax*) contains the *Bax* gene whose expression is controlled by a synthetic GAS4-responsive promoter (GT). The second adenoviral vector contains the *hTERT* (human telomerase reverse transcriptase) promoter with the GT transactivator (Ad/hTERT-GV16). In this binary system, *Bax* expression is induced by expression of a GT transactivator, which is under the control of the *hTERT* promoter. Telomerase is a specialized enzyme involved with the replication of the ends of chromo-

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somes. The enzyme is highly active in immortalized cell lines and more than 85% of human cancers, including pancreatic cancer [11, 12].

In this study, human pancreatic cell lines were transduced with the adenoviral constructs to determine whether delivery of the *Bax* gene is an effective treatment for pancreatic cancer.

MATERIAL AND METHODS

Cell lines. The following human pancreatic cell lines were obtained from the American Type Culture Collection (Rockville, MD): BxPC-3, MIA PaCa-2, and PANC-28. The cells were maintained in RPMI medium supplemented with 5% fetal bovine serum and 1% penicillin and streptomycin (Gibco-BRL, Life Technologies, Inc., Grand Island, NY). All cell lines were incubated at 37°C in a 5% CO_2 incubator.

TRAP assay. The TRAPeze ELISA Telomerase Detection kit (Intergen Co., NY) was used to perform the telomeric repeat amplification. TRAP products were analyzed electrophoretically and with the ELISA kit. The manufacturer's procedures were followed with modifications as specified. Protein concentration was determined by the BCA Protein Assay kit (Pierce, Rockford, IL). A total of 1.0 μg of cell extract was used per assay. The extension products were amplified by PCR with a Thermal Cycler PTC-200 (MJ Research, Inc., Las Vegas, NV). For the electrophoretic analysis, telomerase-specific PCR products were determined by the presence of a DNA ladder (smallest band at 50 bp) on a Novex Pre-cast 10% TBE polyacrylamide gel (Invitrogen, Carlsbad, CA) using the XCell SurelockMini-Cell electrophoresis system (Invitrogen). Gels were stained with SYBR Green I (Molecular Probes, Eugene, OR), and images were captured by a UV transilluminometer (302 nm) and camera system in conjunction with Molecular Analyst software (Bio-Rad Laboratories, Hercules, CA). For telomerase PCR ELISA, TRAP products were quantified by a colorimetric reaction using an Emax Precision microplate reader (Molecular Devices, Sunnyvale, CA).

Recombinant adenovirus vectors. Vectors Ad/GT-*Bax* and Ad/GT-*LacZ* were constructed as previously described [13, 14]. The Ad/hTERT-GV16 vector was constructed by replacing the CMV promoter with a 378-bp hTERT core promoter as previously described [10, 15]. Viral titers were determined by optical absorbance at $A_{260\text{ nm}}$ (one $A_{260\text{ nm}}$ unit = 10^{12} particles/ml) and by plaque assay. Titers determined by $A_{260\text{ nm}}$ (i.e., viral particles) were used in all of the experiments. Particle:plaque ratios normally fell between 30:1 and 100:1. All of the viral preparations were free of contamination by E1+ adenovirus and endotoxin.

Cell proliferation assay. Cells were distributed on 96-well plates at 5×10^3 per well on the day of viral treatment. The cells were synchronized with serum starvation prior to the beginning of the proliferation assay. Cells were transduced with adenoviral vectors at various multiplicities of infection (MOIs). The cells were divided into three groups depending on their treatment: Ad/GT-*LacZ* + Ad/hTERT-GV16, Ad/GT-*Bax* + Ad/hTERT-GV16, and PBS. In each group, the ratio of the two viral vectors was 2:1, a ratio shown to be optimal for the induction of transgene expression in previous experiments [14]. Cell number was determined using CellTiter 96 AQ_{ueous} One Solution Cell Proliferation assay (Promega) at 0-, 24-, 48-, and 72-h time points. All assays were done in quadruplicate.

BAX Western blot analysis. Monolayer cultures of PANC-28 cells were transduced with Ad/GT-*Bax* cells at 5000 MOI for 32 h. Whole cell extracts were prepared in chilled lysis buffer (50 mM Tris–HCl, 150 mM NaCl, 1% NP-40, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 10 μg/ml aprotinin). Cell debris was removed by centrifugation and the supernatants were stored at −20°C. The protein concentration was determined by BCA Protein Assay kit (Pierce). The protein samples

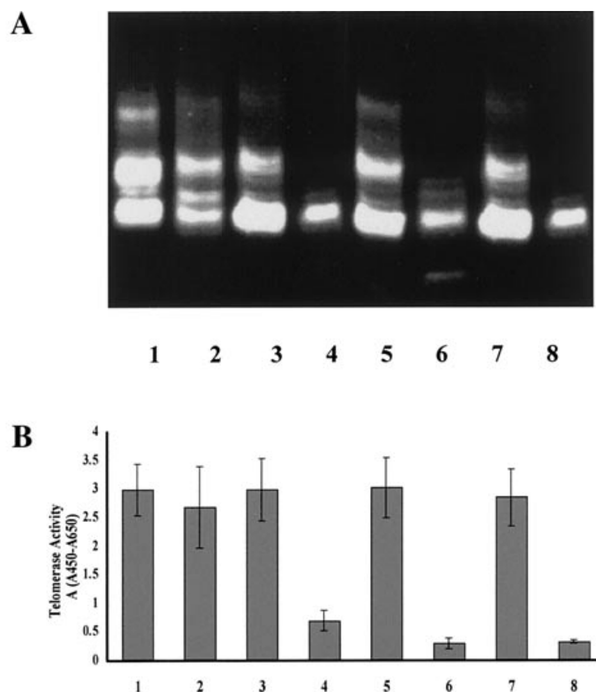

FIG. 1. Telomerase activity in pancreatic cancer cell lines. The human pancreatic carcinoma cell lines BxPC-3, MIA PaCa-2, and PANC-28 all showed evidence of strong telomerase activity by PCR. (A) TBE gels (10%) stained with SYBR Green I showing TRAP reaction signals from control and pancreatic cancer cell extracts. Lane 1 contains the TSR8 control template; lane 2 has the telomerase positive control; lanes 3 and 4 contain the MIA PaCa-2 cell extracts and the heat-inactivated MIA PaCa-2 cells, respectively; lanes 5 and 6 show the PANC-28 telomerase activity and the heat-inactivated PANC-28 cells, respectively; and lanes 7 and 8 show the activity in the BxPC-3 cells and the heat-inactivated BxPC-3 cells, respectively. (B) The TRAP products were quantified by absorbancy reading of the reaction mixtures using ELISA. The results correlate with the telomerase activity observed by gel electrophoresis seen in (A).

were concentrated using Centricon Centrifugal Filter devices (Millipore Co., Bedford, MA). Samples for immunoblotting were prepared by mixing aliquots of the protein extracts with NuPAGE 4× sample (Invitrogen) and 10% β-mercaptoethanol. The samples were then heated for 10 min at 70°C. Protein samples were run on a NuPAGE 10% Bis–Tris gel at 150 V for 1 h and electrotransferred to a PVDF membrane (Millipore Co.) using the Xcell Surelock electrophoresis and transfer apparatus (Invitrogen). The membrane was blocked in 3% casein, TBS solution (Tris-buffered saline and 0.05% Tween 20) overnight at 4°C. The membrane was subjected to immunoblot analysis with 1:250 dilution of *Bax* monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and then with the secondary antibody of horseradish-peroxidase-linked anti-mouse IgG antibody (1:5000 dilution). Anti-actin antibody (1:5000, Sigma Chemical Co.) was used to confirm that equal amounts of protein were loaded. Proteins were visualized by enhanced chemiluminescence (Amersham, Buckinghamshire, UK).

Apoptosis analysis by flow cytometry. PANC-28 cells were distributed onto 100-mm plates 1 day prior to transduction. Cells were then counted and treated with adenoviral vectors at a MOI of 5000 viral particles/cell. Forty-eight hours later, both floating and adherent cells were harvested, pelleted by centrifugation (400 *g* for 5 min), resuspended in Hank's balanced salt solution, and fixed in 70%

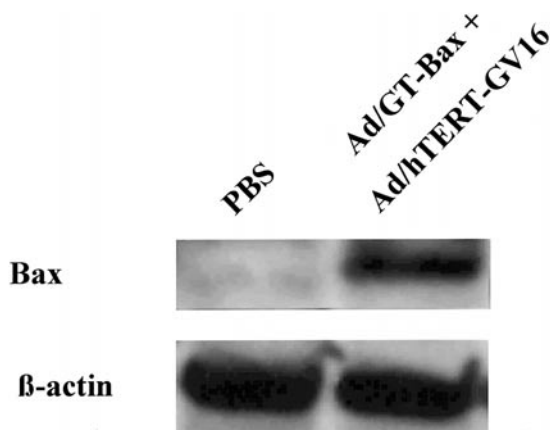

FIG. 2. Overexpression of *Bax* protein following transduction of *Bax* by Ad/GT-*Bax* + Ad/hTERT-GV16 in PANC-28 was documented by Western blotting. β-Actin was used to control for equal protein loading. PBS was used as a control.

ethanol. Apoptosis was quantified using flow cytometry on an Epics Elite flow cytometer (Coulter, Miami, FL).

Cytochrome c Western blot analysis. PANC-28 cells were distributed onto 100-mm plates 1 day prior to infection. They were then counted and treated with adenoviral vectors at a MOI of 5000 viral particles/cell. Twenty-four and 40 h later, floating and adherent cells were harvested through trypsinization. Fractionation of the cytosolic and mitochondrial protein samples was performed using the Apo-Alert cell fractionation kit (Clontech Laboratories, Palo Alto, CA). Protein samples were then concentrated using Centricon centrifugal filter devices (Millipore). Proteins were resolved at 200 V on 10% NuPAGE Bis–Tris gels (Invitrogen) and transferred to nitrocellulose membranes for 1 h at 30 V. Membranes were incubated overnight in blocking buffer (3% casein in TBS) to prevent nonspecific binding. Blots were probed with cytochrome *c* antibody (ApoAlert cell fractionation kit) and were developed using species-specific secondary antibodies and visualized using enhanced chemiluminescence (Amersham Corp., Arlington Heights, IL). Equal protein loading for the cytochrome *c* assay was determined based on protein concentration using the Bio-Rad Protein Concentration assay (Hercules, CA).

Caspase-3 activity assay. Caspase-3 activity was assayed by cleavage of a fluorogenic caspase-3 substrate (DEVD-AFC) using a commercially available kit (Clontech, Palo Alto, CA). PANC-28 cells were treated with Ad/GT-*Bax* + Ad/hTERT-GV16 or Ad/GT-*LacZ* + Ad/hTERT-GV16 at an MOI of 5000 viral particles/cell and harvested after 48 h of incubation. Cells (1×10^6) were incubated with DEVD-AFC (50 μM final concentration) in the presence of DTT for 60 min at 37°C. Fluorometric detection for caspase-3 was performed using a 400-nm excitation and 510-nm emission filter.

Statistical analysis. All growth assays were performed in quadruplicate and differences between treatment groups were analyzed by ANOVA. Differences in caspase-3 levels among treatment groups were analyzed using the *t* test.

RESULTS

Telomerase Activity in Pancreatic Cancer Cells

The human pancreatic carcinoma cell lines BxPC-3, MIA PaCa-2, and PANC-28 all showed evidence of strong telomerase activity by PCR demonstrated by gel electrophoresis and ELISA (Fig. 1). Heat denaturation abolished telomerase activity.

Overexpression of Bax by Western Blotting

Overexpression of *Bax* protein following transduction of *Bax* by Ad/GT-*Bax* + Ad/hTERT-GV16 in PANC-28 was documented by Western blotting (Fig. 2). β-Actin was used to control for equal protein loading.

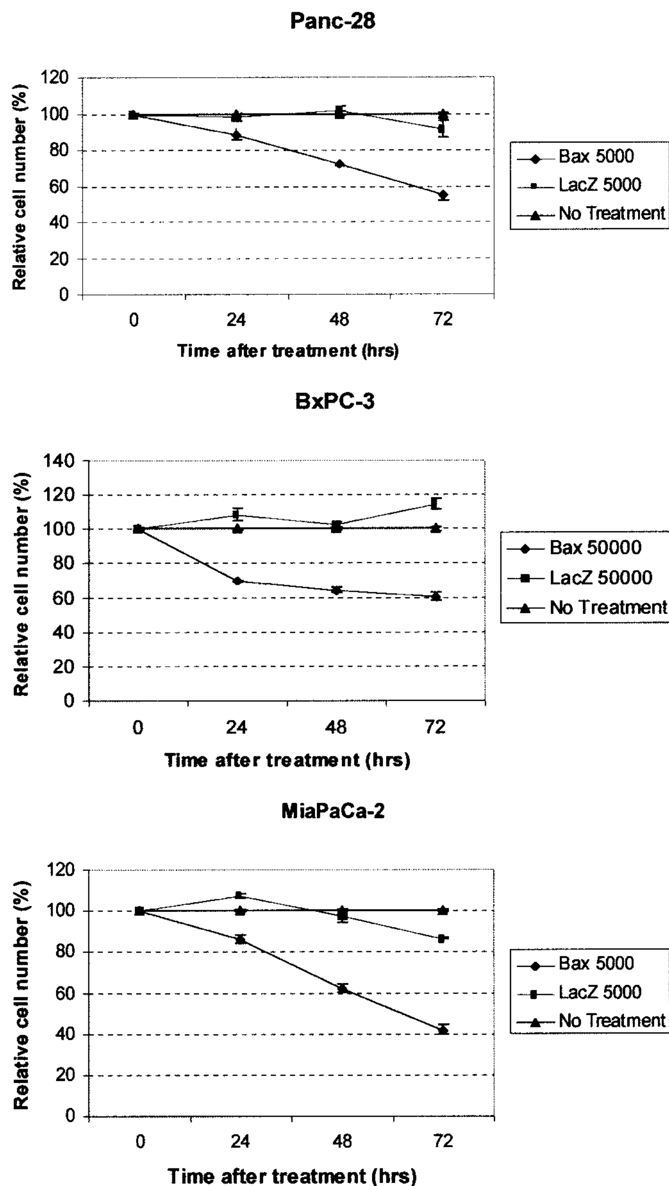

FIG. 3. Effects of *Bax* on cell proliferation. Three different human pancreatic cancer cell lines, BxPC-3, MIA PaCa-2, and PANC-28, were treated with Ad/GT-*Bax* + Ad/hTERT-GV16, Ad/GT-*LacZ* + Ad/hTERT-GV16, or medium alone. Cell proliferation was monitored at 0-, 24-, 48-, and 72-h time intervals after transduction using a cell titer assay (see Material and Methods for experimental details). *Bax* treatment suppressed growth of MIA PaCa-2 and PANC-28 cells when treated with an MOI of 5000 and BxPC-3 with an MOI of 50,000. At these MOIs, few effects were seen with control vector.

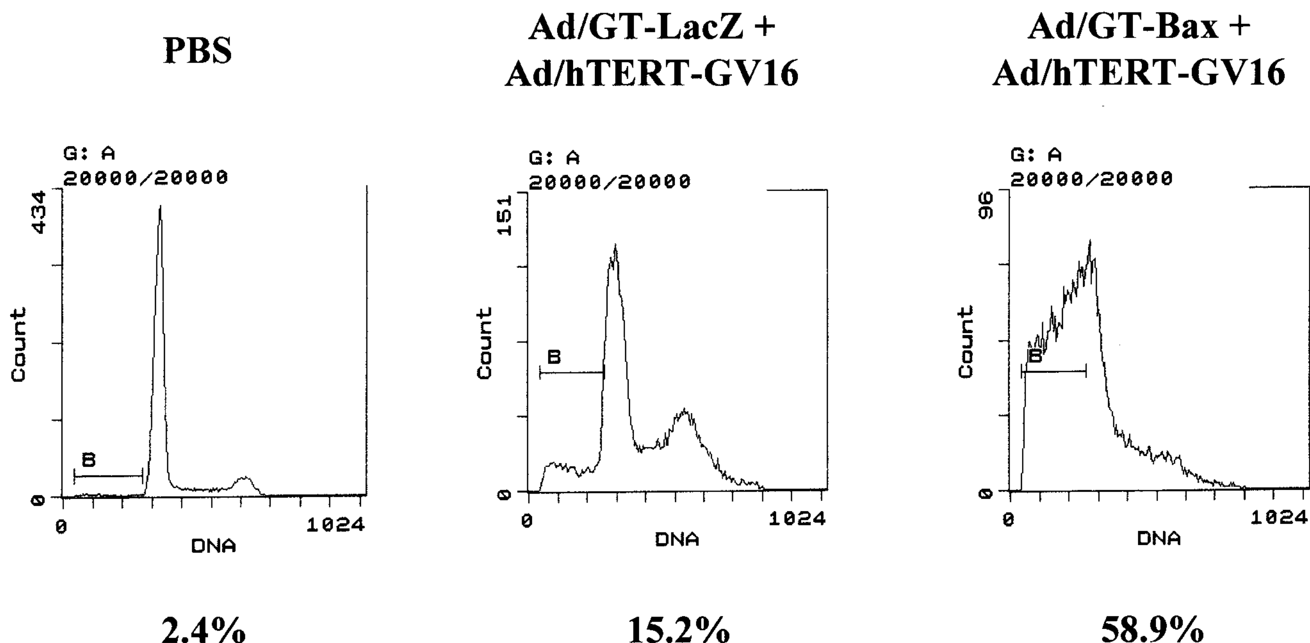

FIG. 4. Introduction of *Bax* into PANC-28 cells induced apoptosis. Increases in fluorescent labeling in the sub-G_1 cell population were observed by FACS analysis in the cells 48 h following infection. Apoptosis was observed in 58.9% of cells treated with *Bax* compared to 15.2% in the vector control or 2.4% in the PBS control cells.

The Effect of Bax on in Vitro Cell Proliferation

Three different human pancreatic cancer cell lines, BxPC-3, MIA PaCa-2, and PANC-28, were treated with Ad/GT-*Bax* + Ad/hTERT-GV16, Ad/GT-*LacZ* + Ad/hTERT-GV16, or medium alone. Cell proliferation was monitored at 0-, 24-, 48-, and 72-h time intervals after transduction. *Bax* treatment suppressed growth of MIA PaCa-2 and PANC-28 cells when treated with an MOI of 5000. In contrast, growth suppression in the BxPC-3 cell line was seen with an MOI of 50,000 (Fig. 3). At these MOIs, few effects were seen with control vector ($P < 0.001$).

The Ability of Bax to Induce Apoptosis

To examine the mechanism of growth suppression in pancreatic cancer by *Bax*, we determined apoptosis by FACS analysis, by caspase-3 levels, and by cytochrome *c* release into the cytosol in PANC-28 cells. Increases in fluorescent labeling in the sub-G_1 cell population were observed by FACS analysis 48 h following transduction of *Bax* into PANC-28 cells (Fig. 4). Apoptosis was observed in 58.9% of cells treated with *Bax* compared to 15.2% vector control or 2.4% of PBS control cells. Introduction of *Bax* into PANC-28 cells caused the release of cytochrome *c* from the mitochondria into the cytoplasm of the cells, also indicating apoptosis (Fig. 5). Caspase-3 levels, as measured by ELISA, were significantly elevated after *Bax* gene therapy compared to controls ($P = 0.03$) (Fig. 6).

DISCUSSION

Long-term survival is disappointing for patients with pancreatic cancer and therefore new treatment modalities are necessary. Currently, many different

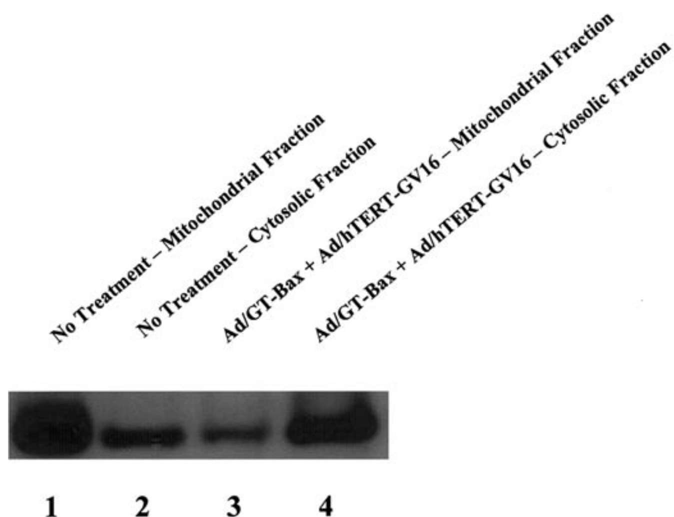

FIG. 5. Cytochrome *c* expression in untreated and treated PANC-28 cells. Lane 1, mitchondrial fraction of untreated cells; lane 2, cytosolic fraction of untreated cells; lane 3, mitochondrial fraction of treated cells; and lane 4, cytosolic fraction of treated cells. See Material and Methods for experimental details. Introduction of *Bax* into PANC-28 cells caused the release of cytochrome *c* from the mitochondria into the cytoplasm of the cells. Release of cytochrome *c* further confirms apoptosis due to *Bax*.

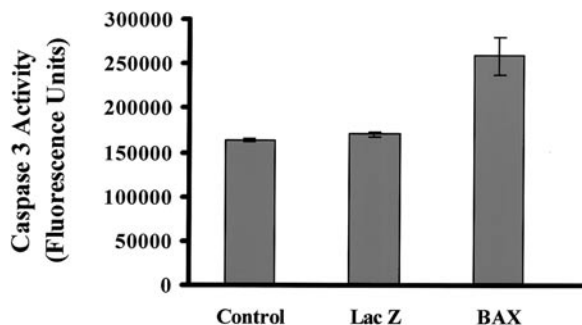

FIG. 6. Caspase-3 induction by *Bax* in PANC-28 cells. Caspase-3 is a key downstream enzyme involved in apoptosis. Caspase-3 levels as measured by ELISA were significantly elevated after *Bax* gene therapy in PANC-28 cells compared to controls ($P = 0.03$).

experimental gene therapy techniques are being evaluated in preclinical models of pancreatic cancer [4]. Adenovirus-mediated transfer of p53 has been shown to cause apoptosis and regression of pancreatic cancer in a mouse model [3]. Transfer of wild-type p16, a gene involved in cell cycle control, produces significant growth suppression of pancreatic cancer *in vitro* and *in vivo* [16]. Adenovirus-mediated anti-K-ras ribozymes have also been used to induce apoptosis of pancreatic tumors [17]. Other studies have also shown tumorlytic effects of treating pancreatic tumors with other viruses such as herpes simplex virus [18, 19]. We have recently shown that methioninase cancer gene therapy with selenomethionine as suicide prodrug substrate is effective against several tumor types including pancreas [20].

Such gene therapy strategies have limitations to their efficacy including a lack of tumor specificity. Recently, investigators have attempted to make gene therapy more selective for tumor cells in order to reduce potential harmful effects to normal tissues. Such strategies take advantage of genes or proteins that are specific to tumor cells. Telomerase is a specialized DNA polymerase responsible for the replication of chromosomal ends, or telomeres. Telomerase is highly active in immortalized cell lines and >85% of human cancers but is inactive in most somatic cells [21, 22]. The enzyme is a ribonucleoprotein complex composed of an essential RNA template and several associated proteins, among which is the essential catalytic subunit named TERT. Because the hTERT gene is highly active in tumor cells but repressed in most normal cells and because its expression is regulated at the transcription level, the hTERT promoter has been used for tumor-specific expression of transgenes including *Bax* [10]. In this study we have shown that telomerase is present in pancreas cancer cells. By taking advantage of this tumor-specific feature, high levels of the *Bax* protein were introduced into pancreatic cancer cells and subsequent growth suppression was induced. We observed extensive apoptosis as documented by FACS analysis, cytochrome *c* migration to the cytosol, and caspase-3 activity with this gene therapy strategy. In conclusion, our results indicate that this binary adenoviral vector system induces high levels of *Bax* and apoptosis in human pancreatic cancer cells, suggesting that this may be a useful gene therapy strategy pancreatic cancer.

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