ADENOVIRUS-MEDIATED BAK GENE TRANSFER INDUCES APOPTOSIS IN MESOTHELIOMA CELL LINES

Abujiang Pataer, MD, PhD^a
W. Roy Smythe, MD^a
Robert Yu, MS^a
Bingliang Fang, MD, PhD^a
Tim McDonnell, MD, PhD^b
Jack A. Roth, MD^a
Stephen G. Swisher, MD^a

Objective: Conventional treatment for mesothelioma is largely ineffective. We therefore evaluated the novel approach of adenoviral gene transfer of the proapoptotic Bcl-2 family member Bak in mesothelioma cancer cell lines, which are sensitive and resistant to adenoviral p53.

Methods: Binary adenoviral Bak (Ad/GT-Bak and Ad/GV16) and LacZ (Ad/GT-LacZ and Ad/GV16) vectors were used for transduction of the mesothelioma cell lines I-45 (p53 resistant) and REN (p53 sensitive). Protein levels were determined by Western blotting. Apoptosis was assessed by morphologic changes, caspase-3 cleavage, and fluorescence-activated cell sorter analysis of subdiploid populations. Cell viability was determined with the XTT assay. Statistical analysis was performed with analysis of variance and the Student *t* test.

Results: High levels of Bak gene transfer were seen after coadministration of Ad/GT-Bak and Ad/GV16 in both mesothelioma cell lines. Apoptosis was induced 24 hours after Bak but not LacZ gene transfer ([Bak: I-45, 36%; REN, 25%] vs [LacZ: I-45, 1%; REN, 3%], $P < .05$) in p53-sensitive (REN) and p53-resistant (I-45) cell lines. Cellular viability was significantly decreased 48 to 72 hours after Bak gene transfer compared with control vector in both cell lines (72 hours: Bak I-45, $1.4\% \pm 1.0\%$, and Bak REN, $4.7\% \pm 1\%$, vs Lac-Z I-45, $83\% \pm 3\%$, and Lac-Z REN, $100\% \pm 1\%$; $P < .05$).

Conclusions: Adenovirus-mediated overexpression of the Bak gene induces apoptosis and decreased cellular viability in p53-sensitive and p53-resistant mesothelioma cells. These data suggest that the gene transfer of proapoptotic Bcl-2 family members may represent a novel gene therapy strategy to treat mesothelioma. (J Thorac Cardiovasc Surg 2001;121:61-7)

Malignant pleural mesothelioma remains a therapeutic problem. Conventional therapy with surgery, chemotherapy, or radiotherapy has largely been ineffective in curing the majority of patients.^{1,2} The advent of gene therapy offers a novel mode of therapy to treat malignant pleural mesotheliomas through the transfer of functional genes. Phase I clinical trials in mesothe-

lioma have demonstrated the feasibility of transferring the herpes simplex virus thymidine kinase (HSVtk) gene followed by treatment with the prodrug ganciclovir.^{3,4} Another gene therapy strategy that has been reported in locoregionally advanced non-small cell lung cancer involves the induction of apoptosis with the transfer of the tumor suppressor *p53* gene.⁵

From the Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery,^a and the Department of Molecular Pathology,^b The University of Texas M.D. Anderson Cancer Center, Houston, Tex.

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Address for reprints: Stephen G. Swisher, MD, Department of Thoracic and Cardiovascular Surgery, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Blvd, Box 109, Houston, TX 77030 (E-mail: sswisher@mdanderson.org).

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Fig 1. Diagram of binary adenoviral vector Ad/GT-Bak, in which *Bak* gene expression is controlled by the presence of Gal4/GV16, requiring coadministration of both vectors to induce *Bak* gene expression.

Apoptosis is a conserved pathway in all cells, which, when activated, leads to cellular death by caspase activation and subsequent chromosome condensation and nuclear degradation.⁶ Many tumor cells have evaded apoptotic cell death by inactivating p53, which is believed to be an upstream initiator of apoptosis.⁷ Replacement of wild-type p53 with gene transfer can lead to apoptosis in lung cancer cells because this allows the apoptotic pathway to be restored. Unlike lung cancer cells, however, many mesothelioma cells fail to undergo apoptosis after p53 gene transfer. The reason for this may be due to the fact that mesothelioma cells have a very low rate of p53 mutations, and therefore gene therapy strategies on the basis of p53 replacement are ineffective because most mesothelioma cells already have functional p53.⁸ We therefore decided to evaluate other proapoptotic genes. The Bcl-2 family is another group of genes important in the regulation of apoptosis with either proapoptotic (Bax and Bak) or antiapoptotic (Bcl-2 and Bcl-X_L) members.⁹ We therefore hypothesized that overexpression of the Bak gene would lead to apoptosis in p53-resistant and p53-sensitive mesothelioma cells by overwhelming antiapoptotic Bcl-2 family members (Bcl-2 and Bcl-X_L) and bypassing upstream p53 resistance. To address this question, we used a binary adenoviral vector system containing 2 adenoviral vectors in which one vector, Ad/GT-Bak, contained the Bak gene under control of the GT promoter and the GAL4/GV16 fusion protein.¹⁰ Bak gene expression could then be induced in target tissues by coadministration of the Ad/GT-Bak vector with the second adenoviral vector Ad/GV16, which produced the GAL4/GV16 fusion protein (Fig 1).

We have previously shown that Ad/GT-Bak can induce apoptosis in p53-sensitive lung cancer cells in a caspase 3-dependent fashion,¹¹ but we were unsure whether it could also induce apoptosis in mesothelioma cells that, unlike lung cancer cells, are mostly p53 resistant. To answer this question, we evaluated two mesothelioma cell lines, one that was p53 sensitive (REN, p53 mutant) and one that was p53 resistant (I-45, p53 wild-type). Our findings showed that we were able to induce apoptosis and decreased cell viability in both p53-resistant and p53-sensitive mesothelioma cell lines, suggesting that the induction of apoptosis and cell death in mesothelioma with Ad/GT-Bak may represent a novel treatment.

Material and methods

Cell lines and antibodies. The REN (inflammatory epithelial subtype, p53 mutant) mesothelioma cell line was developed by one of the authors (W.R.S.) and has been previously described.⁴ The I-45 (sarcomatous subtype, p53 wild-type) cell line was originally provided by Dr Joe Testa of the Fox Chase Cancer Center in Philadelphia, Pennsylvania. All cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum, 10 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Life Technologies, Inc, Grand Island, NY) in a 5% carbon dioxide atmosphere at 37°C. The following antibodies were used: monoclonal antibody to Bcl-X_L was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif). The polyclonal antibody to CPP32 (caspase 3) was purchased from PharMingen (San Diego, Calif). The polyclonal antibodies raised against Bak, Bax, and β-actin were bought from Santa Cruz Biotechnology.

Adenovirus production and transduction. Construction of the Ad/GT-LacZ, Ad/GV16, and Ad/GT-Bak vectors have

Fig 2. Western blot analysis of the expression of Bak, Bax, and Bcl-X_L in cell lysates 24 hours after treatment with PBS (lanes 1), Ad/GT-LacZ (lanes 2), and Ad/GT-Bak (lanes 3). The expression level of actin was used as a control.

been reported previously.^{10,11} The binary adenoviral vector system required coadministration of Ad/GT-Bak and Ad/GV16 (hereafter described as Ad/GT-Bak) or Ad/GT-LacZ and Ad/GV16 (hereafter described as Ad/GT-LacZ). The transduction efficiencies of the cell lines were determined with Ad/GT-LacZ and Ad/GV16. Subsequent experiments used viral titers needed to transduce at least 90% of the cells (MOI, 2400 viral particles = 1600 viral particles [Ad/GT-Bak] + 800 viral particles [Ad/GV16]).

Western blot analysis. Total cell lysates were prepared by lysing cell monolayers in plates with sodium dodecyl-sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Each lane was loaded with 5 µg of cell lysate protein, as determined by BCA protein assay (Pierce, Rockford, Ill). Subsequently, proteins were separated under reducing conditions on an SDS polyacrylamide gel. After electrophoresis at 20 mA for 2 hours, the proteins in the gels were transferred to Hybond-ECL membranes (Amersham Corp, Arlington Heights, Ill). Then the membranes were blocked with 1% dry milk and 0.1% Tween-20 (Sigma, St Louis, Mo) and incubated with primary antibodies. The membranes were then incubated with secondary antibody conjugated with horseradish peroxidase (Amersham, Piscataway, NJ). The membranes were developed according to the Amersham ECL protocol. Relative quantities of protein were determined by using a densitometer (Molecular Dynamics Inc, Sunnyvale, Calif).

Apoptosis analysis. Apoptotic cell death was examined by assessment of cell morphology after Hoechst staining and flow cytometric analysis of cells for propidium iodide exclusion 24 to 48 hours after transduction. Specimens were analyzed in an EPICS Profile II flow cytometer (Coulter Corp, Hialeah, Fla). Additionally, caspase cleavage was assessed by means of Western blotting to determine activation of the execution phase of apoptosis.

Cellular viability analysis. Cells were grown in 96-well plates in 100 µL volume per well. On the first day, cells were transduced with Ad/GT-Bak or control vectors. On the second day, the cells were incubated with the tetrazolium salt XTT for 4 to 24 hours, according to the Roche protocol (Roche Diagnostics, Mannheim, Germany). Viability was assessed spectrophotometrically on an enzyme-linked immunosorbent assay (ELISA) plate reader.

Statistical analysis. Data in the figures represent the mean of 3 independent experiments with the SD. Analysis of variance and the 2-tailed Student *t* test were used for statistical analysis.

Results

Transduction of mesothelioma cells by Ad/GT-Bak and Ad/GV16. The mesothelioma cell lines used in these experiments included cells with null p53 (REN) and wild-type p53 (I-45) genotypes. The titers used for these experiments were determined by experiments with Ad/GT-LacZ, which demonstrated that the binary adenoviral vector system achieved over 90% transduction efficiencies with 2400 viral particles (1600 Ad/GT-Bak plus 800 Ad/GV16). Only low levels of endogenous Bak protein were observed in cells treated with phosphate-buffered saline solution (PBS) or infected with Ad/GT-LacZ, whereas high levels of Bak protein were seen as early as 24 hours after transduction with Ad/GT-Bak (Fig 2). The levels of proapoptotic Bax and antiapoptotic Bcl-X_L did not change after viral transduction with either control or Ad/GT-Bak vectors (Fig 2).

Induction of apoptosis in mesothelioma cells by Ad/GT-Bak. Transduction of the mesothelioma cell lines with Ad/GT-Bak induced morphologic changes

Fig 3. Hoechst staining of mesothelioma cells 24 hours after treatment with PBS (A), Ad/GT-LacZ (B), or Ad/GT-Bak (C). Only the cells treated with Ad/GT-Bak showed the presence of cytoplasmic contraction, membrane blebbing, and the formation of apoptotic bodies.

Table I. Induction of apoptosis in mesothelioma cell lines after Ad/GT-Bak transduction

Mesothelioma cell line	Apoptosis			
	PBS control*	Ad/GT-LacZ*	Ad/GT-Bak*	Adenoviral p53*
I-45 (p53 wild-type)	1% $\pm$ 1%	1% $\pm$ 1%	36% $\pm$ 3%†	5% $\pm$ 2%
REN (p53 mutant)	2% $\pm$ 1%	3% $\pm$ 1%	25% $\pm$ 4%†	30% $\pm$ 5%†

*Mean of 3 experiments $\pm$ SD.

† $P < .05$ accepted as statistically significant.

within 24 hours. Cells treated with Ad/GT-Bak became shrunken and dense appearing, often detaching from the cell plates, whereas cells from the other control groups remained in monolayers with normal morphology. Hoechst staining was performed and revealed the presence of cytoplasmic contraction, membrane blebbing, and the formation of apoptotic bodies in the Ad/GT-Bak-treated cells (Fig 3). To determine whether these changes were due to apoptosis, propidium iodide staining and fluorescence-activated cell sorter analysis was performed, looking for subdiploid populations. As Table I shows, Ad/GT-Bak treatment induced apoptosis in both the p53-sensitive (REN) and p53-resistant (I-45) cell lines as early as 24 hours after transduction. Additional evidence for the induction of apoptosis by Ad-GT-Bak included demonstration of caspase 3 cleavage from the inactive proenzyme to the activated 17-kd fragment, which is associated with onset of the execution phase of apoptosis (Fig 4). Cleavage of the caspase

3 proenzyme was noted in both mesothelioma cell lines and was not seen in cells treated with control vector (Ad/GT-LacZ) or PBS.

Decreased cellular viability in mesothelioma cells after Ad/GT-Bak. Cellular viability was assessed with the XTT assay. Transduction of both the p53-sensitive (REN) and p53-resistant (I-45) mesothelioma cell lines resulted in marked decrease in cellular viability 48 to 72 hours after infection (Fig 5). The REN (mutant p53) demonstrated 18% $\pm$ 3% viability at 48 hours and 4.7% $\pm$ 1% at 72 hours. The I-45 (wild-type p53) cell line showed only 7.7% $\pm$ 2.5% viability at 48 hours and 1.4% $\pm$ 1% at 72 hours. Treatment with control vector resulted in no decreased viability in REN cells but did note some decrease at 72 hours in I-45 cells 83% $\pm$ 3%.

Discussion

Malignant pleural mesothelioma is a tumor that is unresponsive to standard modalities of cancer treat-

Fig 4. Western blot analysis of the expression of Bak and caspase 3 in cell lysates 24 hours after treatment with PBS (lanes 1), Ad/GT-LacZ (lanes 2), and Ad/GT-Bak (lanes 3). The expression level of actin was used as a control.

Fig 5. XTT assessment of cellular viability 24 to 72 hours after transduction of I-45 (A) or REN mesothelioma cells (B) with PBS, Ad/GT-LacZ, or Ad/GT-Bak.

ment.^{1,2} Gene therapy offers the opportunity of a new modality of therapy for this fatal disease. A recently completed phase I clinical trial in mesothelioma demonstrated the feasibility of treating malignant mesothelioma with intrapleural instillation of herpes simplex virus thymidine kinase (HSVtk).³ In that study 21 patients were treated with minimal toxicity with a replication-incompetent adenoviral vector containing the gene encoding HSVtk. Other gene therapy strategies include the induction of apoptosis with the transfer of the tumor suppressor *p53* gene. One recently completed trial in non-small cell lung cancer demonstrated clinical responses with adenovirus-mediated overexpression of wild-type *p53* in patients in whom all conventional treatments had failed.⁵ It is clear, however, that not all patients respond to adenoviral *p53*. Additionally, preclinical experience with mesothelioma cell lines have demonstrated *p53* resistance in a number of cell lines. We therefore evaluated the effectiveness of adenoviral transfer of the proapoptotic Bak gene in mesothelioma cell lines.

The induction of apoptosis or programmed cell death reflects a balance between signaling events and molecules that either promote or inhibit apoptosis.^{6,12} Current data support the hypothesis that within the Bcl-2 family, the ratio of death antagonist to agonist determines whether a cell will respond to apoptotic signals.¹³ This hypothesis favors the use of adenoviral vectors because they are able to induce high levels of gene expression, which would presumably overwhelm the levels of antiapoptotic Bcl-2 members. Our observations support this hypothesis because Ad/GT-Bak was able to induce apoptosis, even in the presence of high endogenous levels of antiapoptotic Bcl-X_L (Fig 2).

Our previous study demonstrated that adenovirus-mediated overexpression of Bak could induce apoptosis in *p53*-sensitive lung cancer cells.¹¹ This study demonstrated the novel observation that Ad/GT-Bak could also induce apoptosis in cells that are resistant to *p53*. The observation that *p53*-resistant mesothelioma cell lines are sensitive to Ad/GT-Bak is not necessarily expected because *p53* works in part by up-regulating Bak and Bax.^{14,15} It may be that the very high tissue levels of Bak achieved with adenoviral vectors allows adenoviral Bak gene transfer to be effective. Additionally, Bak may be acting downstream from *p53* in the apoptotic cascade, thus allowing it to bypass a genetic blockage further upstream in the cascade. It is important to note, however, that even though Bak gene transfer is not *p53* dependent, the process appears caspase dependent because cell lines that lack functional caspase 3 are resistant to

Parp cleavage and development of subdiploid populations by fluorescence-activated cell sorter analysis.¹¹

The increased apoptotic potential of Ad/GT-Bak may come at a price, however, because we have observed that Ad/GT-Bak appears less selective than adenoviral *p53* and more likely to induce apoptosis in normal human bronchial epithelial cells (data not shown). Clinical strategies to overcome toxicity to normal tissue include tumor selective promoters, such as the telomerase or DF3 promoter.^{10,16} Alternatively, as experience with HSVtk demonstrates, direct intrapleural instillation may allow proportionally more mesothelioma tumor cells to be transduced, thereby minimizing toxicity to normal tissue.

In summary, we have shown that adenovirus-mediated overexpression of Bak leads to rapid apoptosis in mesothelioma cells. This antitumor strategy does not appear dependent on *p53* sensitivity, *p53* genetic status, or level of antiapoptotic Bcl-X_L. The lack of selectivity for normal and cancer cells may require strategies, such as tumor selective expression or intrapleural localization, to avoid toxicity to normal tissues. Because this adenovirus-based strategy does not require *p53* and induces apoptosis in *p53*-resistant tumors, it may ultimately provide a novel gene therapy strategy for patients with malignant pleural mesothelioma.

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Discussion

Dr Nasser K. Altorki (*New York, NY*). Dr Swisher, I have a couple of questions for you. Correct me if I am wrong, but I had the impression that the bcl-2-mediated apoptotic mechanism is also to some extent p53 dependent. Is that not so?

Dr Swisher. You are quite right. p53 appears to induce apoptosis through a cascade with p53 at the top, inducing other proapoptotic genes, such as proapoptotic Bcl-2 family members like Bak, which then induce activated caspases to dismantle the cell. Our interest in the Bcl-2 family was that we thought if there were functional problems with p53 up higher, then perhaps we could induce apoptosis further down the cascade through the Bcl-2 family and overcome some of the p53 resistance noted in mesothelioma cells. In fact, we have previously shown that transduction of lung cancer cells

with adenoviral p53 leads to elevated levels of proapoptotic Bak and Bax, which may be responsible for the induction of apoptosis seen after p53 gene transfer.

Dr Altorki. Obviously, this is an impressive piece of work. How do you envision doing that in a model of mesothelioma? It seems to me that the delivery may not be quite as simple.

Dr Swisher. There are obviously 2 problems in translating adenoviral Bak gene therapy to the clinical arena in mesothelioma. The first problem is the preclinical one, which we have looked at in this study, and that is how apoptosis in mesothelioma cells is induced. The second problem is how this can be effective clinically. I agree that one of the biggest problems with gene transfer in mesothelioma is the difficulty of delivering the vector to the pleural surface because of the loculations within the hemithorax. Therefore, I would envision delivering the vector in conjunction with an operation, perhaps after a pleural pneumonectomy, when the gross disease and loculations have been removed, to get those residual small cells that are remaining.

Dr David J. Sugarbaker (*Boston, Mass*). I have one brief question that Dr Altorki touched on. With the data now showing that improved local control of mesothelioma in human subjects undergoing either pleural pneumonectomy or other more radical operations, we are beginning to see systemic metastases, more so than in the past. Can you speculate as to how you might deliver this system systemically to affect not just the hemithorax but also a systemic distribution?

Dr Swisher. One of the problems with adenoviral gene transfer is trying to target systemic micrometastases. You can get high levels of expression intratumorally with direct injection, but it is difficult to get high levels systemically through the bloodstream. I think there may be potential in the future to address systemic micrometastases with liposomes. In addition, some work has been done recently to target these liposomes to cell surface receptors. I notice that an upcoming presentation in this forum documents high levels of folate receptors in mesothelioma cells, and one can target the liposomes to these receptors. Theoretically, with a liposomal targeting system, one may be able to address systemic micrometastases in the future.

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