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Supported by grants from the National Cancer Institute and the NIH (Grant P01 CA78778-01A1; to J. A. R.), by Specialized Program of Research Excellence in Lung Cancer (Manassas, VA), the American Lung Association, and the American Lung Association of MD Anderson Cancer Center. The human breast carcinoma cell line MCF-7 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA). The human lung cancer cell line A549 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA). The human lung cancer cell line H1299 (null p53), A549 (wild-type p53), and H460 (wild-type p53), and A549 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA).

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The abbreviations used are: PARP, pol(ADP ribose) polymerase; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; GFP, green fluorescence protein.

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3 One of the most promising strategies in cancer gene therapy is adenoviral transfer of proapoptotic genes. We therefore evaluated the novel strategy of adenovirally overexpressing the proapoptotic Bak gene. Our results showed marked apoptosis in cancer cells in vivo and in vitro after Bak gene transfer via a binary adenoviral vector system. This effect was not seen in a caspase 3-defective cell line (MCF-7) and was abrogated in Bak-sensitive tumors after administration of the caspase inhibitor z-DEVD-fmk. Our results suggest that adenosil-mediated overexpression of Bak provides a novel therapeutic strategy for cancer therapy, but this process appears to be caspase dependent.

Abstract

One of the most promising strategies in cancer gene therapy is adenoviral transfer of proapoptotic genes. We therefore evaluated the novel strategy of adenovirally overexpressing the proapoptotic Bak gene. Our results showed marked apoptosis in cancer cells in vivo and in vitro after Bak gene transfer via a binary adenoviral vector system. This effect was not seen in a caspase 3-defective cell line (MCF-7) and was abrogated in Bak-sensitive tumors after administration of the caspase inhibitor z-DEVD-fmk. Our results suggest that adenosil-mediated overexpression of Bak provides a novel therapeutic strategy for cancer therapy, but this process appears to be caspase dependent.

Introduction

The Bcl-2 family of genes appears to be important in the regulation of apoptosis. Members of this family are cellular homologues that are either proapoptotic (Bax and Bak) or anti-apoptotic (Bcl-2 and Bcl-XL; Ref. 1). Our initial attempts to develop an adenoviral vector containing the Bak gene were complicated by high levels of apoptosis induced in the packaging 293 cell line. This problem was overcome by our development of a binary adenoviral vector system containing two adenoviral vectors. One of the two adenoviral vectors, Ad/GT-Bak, contains a Bak gene under the control of the GT promoter and the GAL4/GV16 fusion protein. This Ad/GT-Bak vector can be grown to large quantities in 293 cells without toxicity to the packaging cell line. Bak gene expression can then be induced in target tissues by coadministration of the Ad/GT-Bak vector with the second adenoviral vector in our system, Ad/GV16 (which produces the GAL4/GV16 fusion protein).

Using this novel system, we have found that overexpression of the Bak gene induces high levels of apoptosis in p53 wild-type, mutant, and null tumor cell lines, regardless of the level of the antiapoptotic members of the Bcl-2 family (i.e., Bcl-2 and Bcl-XL). This effect was seen in vitro and in vivo and was accompanied by marked tumor regression of lung cancer cells in a nude s.c. tumor model. Interestingly, despite high levels of transduction, Bak overexpression did not result in apoptosis in a caspase 3-deficient cell line (MCF-7). Furthermore, the addition of the caspase inhibitor z-DEVD-fmk abrogated Bak-mediated tumor killing in tumor cell lines that were previously Bak sensitive. Together, these results suggest that adenosil-mediated overexpression of the proapoptotic Bak gene is indeed a novel strategy for treating tumors, although the mechanism appears to be caspase dependent.

Materials and Methods

Cell Lines, Reagents, and Antibodies. The human lung cancer cell lines H1299 (null p53), H322J (mutant p53), H460 (wild-type p53), and A549 (wild-type p53) were obtained from the American Type Culture Collection (Manassas, VA). The human breast carcinoma cell line MCF-7 (wild-type p53) were obtained from Dr. Kelly Hunt (The University of Texas M. D. Anderson Cancer Center, Houston, TX). The caspase inhibitor z-DEVD-fmk was obtained from Calbiochem (San Diego, CA). The following antibodies were used: (a) monoclonal antibody to Bcl-2 (Santa Cruz Biotechnology, Santa Cruz, CA); (b) monoclonal antibody to PARP (PharMingen, San Diego, CA); (c) polyclonal antibody to caspase 3 (PharMingen); and (d) polyclonal antibodies to BCLX_L, Bak, and β-actin (Santa Cruz Biotechnology).

Adenovirus Production. Construction of the Ad/GT-LacZ and Ad/GV16 vectors was reported previously (2). Ad/CMV-GFP (described hereafter as Ad/GFP) was obtained from Dr. T. J. Liu (Department of Head and Neck Surgery, University of Texas M. D. Anderson Cancer Center, Houston, TX). The Ad/GT-Bak vector was constructed by placing Bak cDNA (obtained from T. J. M.) downstream of the GAL4/TATA promoter (GT) to generate the shuttle plasmid pAd/GT-Bak. This plasmid was cotransfected into 293 cells along with a 35-kb ClaI fragment purified from human adenoviral type 5 to generate the Ad/GT-Bak vector. The transduction efficiencies of adenoviral vectors in various cancer cell lines were determined by infecting cells with Ad/GT-LacZ and then determining the titers needed to transduce at least 80% of the cells.

Flow Cytometry Analysis. Apoptotic cell death was examined in terms of changes in cell morphology and flow cytometric analysis of cells for propidium iodide exclusion and by TUNEL assay 24–48 h after transduction. Specimens were analyzed in an EPICS Profile II flow cytometer (Coulter Corp., Hialeah, FL).

Western Blot Analysis. Total cell lysates were prepared by lysing plated cell monolayers with SDS-PAGE sample buffer. The proteins were transferred to Hybond enhanced chemiluminescence membranes (Amersham Corp., Arlington Heights, IL) and incubated with the primary and secondary antibodies according to the Amersham enhanced chemiluminescence protocol.

In Vivo Tumor Growth and Ad/Bak Treatment. H1299 and A549 cells (5 × 10^5 cells/0.2 ml) were injected s.c. into the flanks of female, athymic, 5–6-week-old, nude mice. Once tumors grew to approximately 5 mm × 5 mm, they were then injected directly with Ad/GT-Bak + Ad/GV16, control vector Ad/GT-Bak + Ad/GFP, control vector Ad/GT-LacZ + Ad/GV16, or PBS. Each injection was given through a single pass of a 31-gauge hypodermic needle. A second virus injection was given 3 days after the first injection. A third virus injection was given 3 days after the second injection. Tumor volume was calculated by assuming a spherical shape and using the formula volume = (a × b^2)/2, where a and b are the maximum and minimum diameters, respectively. Results for six to seven animals from each treatment group were averaged and expressed as the mean ± SD.

To evaluate apoptosis, tumors were removed 12 h after the third injection of virus in vivo, fixed in 10% formalin, embedded in paraffin blocks, and then assessed by histological analysis and TUNEL assays. For TUNEL assay,
sections were dewaxed, rehydrated (55°C for 15 min), washed in xylene, and then rehydrated through a graded series of ethanol and redistilled water. Tissue sections were then incubated with protease K, permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, and labeled with the TUNEL reaction mixture.

Statistical Analysis. ANOVA and a two-tailed Student’s t test were used for statistical analysis when appropriate. Significance was set at $P < 0.05$.

Results

Efficient Transduction of Tumor Cells by Ad/GT-Bak and Ad/GV16 Vectors. Using a binary adenoviral vector system to avoid the toxic effects of Ad/Bak on 293 packaging cells, we successfully produced large amounts of Ad/GT-Bak, whose gene product (Bak) was under the transcriptional control of the GT promoter and the GAL4/GV16 fusion protein. This novel system worked because Bak gene production was silenced without the GAL4/GV16 fusion protein. In vitro or in vivo expression of Bak was then induced by coadministration of Ad/GT-Bak and Ad/GV16, which produced the GAL4/GV16 fusion protein. Using a binary adenoviral LacZ vector system (Ad/GT-LacZ + Ad/GV16), transduction efficiency (i.e., the titer required to achieve greater than 80% transduction) was determined for all cell lines and then used in subsequent experiments (data not shown). Using these titers, high levels of Bak were induced when Ad/GT-Bak + Ad/GV16 was administered, but not when Ad/GT-LacZ or Ad/GFP was administered (Fig. 1, A and B).

Caspase-dependent Induction of Apoptosis by Ad/GT-Bak. We then evaluated the ability of Ad/GT-Bak to induce apoptosis in vitro. As Fig. 1C demonstrates, Ad/GT-Bak induced high levels of apopto-

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Fig. 1. Adenoviral-mediated overexpression of Bak induces cell death. A, Western blot analysis of the expression of Bak and the following antiapoptotic Bcl-2 family members: Bcl-2 and Bcl-XL, in cell lysates 24 h after treatment with PBS (Lanes 1); Ad/GT-Bak + Ad/GFP (Lanes 2); Ad/GT-Bak + Ad/GV16 (Lanes 3); and Ad/GT-LacZ + Ad/GV16 (Lanes 4). The expression level of actin was used as a control. B, Western blot analysis of the expression of Bak, PARP, and caspase 3 proteins in cell lysates 24 h after treatment with PBS (Lanes 1), Ad/GT-Bak + Ad/GFP (Lanes 2), Ad/GT-Bak + Ad/GV16 (Lanes 3), and Ad/GT-LacZ + Ad/GV16 (Lanes 4). The expression level of actin was used as a control. C, percentage of subdiploid cells in H1299 (null p53), H322J (mutant p53), H460 (wild-type p53), A549 (wild-type p53), and MCF-7 (wild-type p53) cells after treatment with PBS, Ad/GT-Bak + Ad/GFP, Ad/GT-Bak + Ad/GV16, and Ad/GT-LacZ + Ad/GV16. The cells were analyzed by flow cytometry 24 h after transfection. Triplicate experiments were performed for each cell line, and data represent the means ± SD.
sis in vitro in all cell lines except MCF-7 as early as 24 h after transduction. The mock-infected (PBS) or virus control-infected (Ad/GFP and Ad/GT-LacZ) cells demonstrated no apoptosis (1–3%), despite being infected with similar viral titers. The Ad/GT-Bak-infected cells, however, demonstrated 40–60% apoptosis 24–48 h after infection (Fig. 1C). This apoptosis was confirmed by characteristic morphological changes, PARP cleavage, and caspase activation (Fig. 1B). PARP and caspase 3, which are useful as indices of apoptosis, were only cleaved in cells that had been transduced with Ad/GT-Bak + Ad/GV16. MCF-7 cells were the only cells that failed to undergo apoptosis, despite adequate Bak transduction (Fig. 1, B and C). Interestingly, the levels of antiapoptotic Bcl-2 family members did not appear to be greater in the MCF-7 cells than in the Bak-sensitive cell lines (Fig. 1A), suggesting that these proteins were not the cause of the resistance in MCF-7 cells. As Fig. 1B demonstrates, however, MCF-7 cells differed from the other cell lines in lacking a functional caspase 3. Additionally, no PARP cleavage was noted in MCF-7 cells after Ad/GT-Bak infection, possibly because of the caspase 3 deficiency.

To test whether Ad/GT-Bak-induced apoptosis and PARP cleavage were caspase dependent, we infected H1299 tumor cells with Ad/GT-Bak in the presence or absence of the caspase blocker z-DEVD-fmk. As Fig. 2A shows, Bak-induced apoptosis was completely abrogated by caspase inhibition. Additionally, PARP cleavage was blocked by the addition of z-DEVD-fmk, despite Bak overexpression (Fig. 2B).

Fig. 3. In vivo regression of tumors after intratumoral injection of Ad/GT-Bak in nu/nu mice. The growth of H1299 tumors was significantly inhibited after injection with Ad/GT-Bak + Ad/GV16 as opposed to PBS, Ad/GT-Bak + Ad/GFP, or Ad/GT-LacZ + Ad/GV16. In each case, mice received a total of three injections over 6 days. Tumor volumes (vertical axis) were measured in 6–7 mice/group over a 20-day period, and data represent the means ± SD.
These results suggest that: (a) MCF-7 cells may be resistant to Ad/GT-Bak killing because they are caspase deficient; and (b) Ad/GT-Bak-mediated tumor killing is caspase dependent.

Tumor Regression Mediated by Ad/GT-Bak in an Animal Model. We then evaluated the ability of Ad/GT-Bak to induce tumor regression in vivo in a s.c. nu/nu tumor mouse model as described in “Materials and Methods.” The Ad/GT-Bak-injected tumors were significantly smaller than both the saline control and virus-control infected tumors (P < 0.05; Fig. 3). In addition, these observations held true in both p53-null (H1299) and p53 wild-type (A549; data not shown) cells, suggesting that the mechanism was p53 independent.

To determine whether this effect was caused by the induction of apoptosis, we isolated tumors 12 h after the initial viral treatment, sectioned them, and subjected them to histology and TUNEL assays. Compared with the tumors of control mice, Ad/GT-Bak-injected tumors were smaller (Fig. 4A), demonstrated more of the cellular debris and morphological changes associated with apoptosis (Fig. 4B), and demonstrated increased TUNEL staining (Fig. 4C). In contrast, treatment with Ad/GT-LacZ or Ad/GFP yielded no histologically detectable apoptotic responses. Additionally, intratumoral injection of Ad/GT-Bak caused no significant systemic toxicity. Together, these results demonstrated the potential antitumor effect of Ad/GT-Bak when directly injected into tumors.

Discussion

Several recent clinical trials have demonstrated the feasibility of using adenovirally mediated gene transfer to treat lung and head and neck cancers (3, 4). In these trials adenoviral-mediated overexpression of wild-type p53 has produced clinical responses in heavily pretreated patients. It is clear, however, that not all patients respond to adenoviral p53, and in some patients who initially respond, the disease ultimately progresses and fails to respond to readministration of adenoviral p53. Therefore, there is a clinical need to identify other candidate genes for use in treating cancer patients with adenoviral vectors. One promising group is the proapoptotic members of the Bcl-2 family (Bax and Bak), which have been shown to induce apoptosis after gene transfer via plasmid vectors in vivo (5, 6).

To our knowledge, ours is the first report of an antitumoral effect of adenovirally mediated overexpression of the proapoptotic Bak gene. Bak is located on chromosome 6 and shares homology with the entire Bcl-2 family, including its antiapoptotic and proapoptotic members (7). In fact, the family members interact through highly conserved areas of Bcl-2 homology (BH1, BH2, and BH3) that allow hetero- and homodimerization and the consequent close regulation of apoptosis. In the case of Bax, this scheme has been well worked out in a rheostat model in which excess proapoptotic Bax suppresses Bcl-2 and induces apoptosis via cytochrome c (8). Less is known about Bak, but there is evidence that Bak functions in a similar manner (7, 9, 10). If true, such a mechanism of action would favor the use of adenoviral vectors because high levels of proapoptotic Bak would presumably be able to overwhelm the levels of antiapoptotic Bcl-2 members, inducing the cell to undergo programmed cell death. Our study suggests that this may indeed be the case because we found that overexpression of Bak was able to induce apoptosis in cancer cells even in the presence of high levels of antiapoptotic Bcl-2 family members (Fig. 1A).

The one cell line that was resistant to Ad/GT-Bak killing was MCF-7, although high levels of Bak protein were induced in such cells (Fig. 1, A–C). The reason for this seeming contradiction may be that the MCF-7 cell line lacks a functional caspase 3 as a result of a genomic mutation that introduces a premature stop codon into the caspase 3 mRNA (11). In the present case, the fact that the caspase inhibitor z-DEVD-fmk completely abrogated Ad/GT-Bak induced apoptosis in cell lines that were previously Bak sensitive supports the hypothesis that Ad/GT-Bak-mediated tumor killing is caspase dependent (Fig. 2, A and B). Unlike Bax, which has been reported to have caspase-dependent and -independent pathways of activation, our studies suggest that Bak-induced apoptosis is predominantly caspase dependent (12). In addition, our studies suggest that this process is...
p53 independent because Bak-induced apoptosis occurred equally well in p53 wild-type, null, or mutant cells. This observation may be explained by the fact that the Bcl-2 family members function downstream from p53 in the apoptotic cascade. Indeed, we have observed that Ad-p53 appears to induce apoptosis in part by up-regulation of Bak and Bax and subsequently inducing apoptosis, perhaps by caspase activation (13). These observations are important because if Bak functions downstream from p53, then the clinical use of the Bak vector may be independent of p53. If so, then treatment of p53 mutant or -resistant cancer cells with adenoviral Bak might be possible.

In summary, we have shown for the first time that adenoviral-mediated overexpression of Bak by Ad/GT-Bak leads to rapid apoptosis of cancer cells in vitro and sustained tumor regression in vivo. This antitumor strategy does not appear to be dependent on p53 status and appears to work even in the presence of high levels of antiapoptotic Bcl-2 family members. The mechanism of action of Bak-mediated apoptosis, however, appears to be caspase dependent. In short, this study suggests that adenoviral overexpression of Bak and other proapoptotic Bcl-2 family members, despite the dependence on caspase, is a novel strategy for cancer gene therapy and is worthy of further exploration.

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References

Adenoviral Bak Overexpression Mediates Caspase-dependent Tumor Killing

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