Abstract

Human telomerase reverse transcriptase (hTERT) is the catalytic subunit of telomerase, which is highly active in immortalized cells and >85% of human cancers but is quiescent in most normal somatic cells. To test the feasibility of using the hTERT promoter to induce tumor-specific transgene expression in cancer gene therapy, we constructed an adenoviral vector expressing a LacZ reporter gene driven by the hTERT core promoter and evaluated its activity in vitro and in vivo. The hTERT promoter could drive high-level expression of LacZ in tumor cells but not in normal cells and normal mouse tissues. Using a binary adenoviral system that can induce Bax gene expression, we showed that induction of the Bax gene expression via the hTERT promoter elicited tumor-specific apoptosis in vitro, suppressed tumor growth in nude mice, and prevented the toxicity of the Bax gene in vitro and in vivo. Thus, the hTERT promoter is apparently a strong and tumor-selective promoter with potential application in targeted cancer gene therapy.

Introduction

Control of gene expression via tissue- or cell-specific promoters has been tested extensively as a means of targeting transgene expression. Several promoters have been identified that are more active in particular tumor types than in the tissues from which they arise, and these promoters have been exploited to target transgene expression in tumors. These promoters include the tyrosinase gene promoter in melanomas (1), the carcinoembryonic antigen (CEA) promoter in colorectal and lung cancer cells (2), the MUC1 promoter in breast cancer (3), and the E2F promoter in cancers that carry a defective retinoblastoma gene (4). Nevertheless, although the reports on these promoters suggest that achieving relatively tumor-specific transgene expression is possible, they also reveal several limitations. First, most of these promoters are limited to specific tumor histologies and cannot be used universally in tumors of various origins. Second, most of these promoters are much weaker than commonly used viral promoters such as the CMV3 early promoter, the Rous sarcoma virus long terminal repeat (RSV-LTR), and the SV40 early promoter. Consequently, their uses are hampered by the problem of low expression.

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 (5, 6). 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. Recently, the hTERT gene has been cloned by several independent groups and it has been found to be expressed at high levels in primary tumors and cancer cell lines but repressed in most somatic tissues (7–10). Recent data suggest that the hTERT is a key determinant of the telomerase activity. This includes the finding that hTERT expression is highly correlated with telomerase activity (7–10) and that the ectopic expression of hTERT in telomerase-negative cells is sufficient to reconstitute telomerase activity (11–13) and extend the life span of normal human cells (14). More recently, Hahn et al. (15) showed that ectopic expression of hTERT is required, but is not sufficient, for direct tumorigenic conversion of normal human epithelial and fibroblast cells.

The promoter region of the hTERT gene has also been cloned recently (16–18). The promoter is highly G/C-rich and lacks both TATA and CAAT boxes but contains binding sites for several transcriptional factors, including Myc and Sp1. Deletion analysis of the hTERT promoter identified a core promoter region of ~200 bp upstream of the transcription start site. Transient expression assays revealed that the core promoter is significantly activated in cancer cell lines but is repressed in normal primary cells.

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, we hypothesized that the hTERT promoter may be used for tumor-specific expression of transgenes. In the present study, we have assessed the capability of adenovirus-mediated transgene expression induced by the hTERT promoter and the feasibility of targeting the pharmaceutical effects of the Bax gene, a strong pro-apoptotic gene, to tumors by this promoter both in vitro and in vivo.

Materials and Methods

Construction of Recombinant Adenovirus Vectors. Vectors Ad/E1−, Ad/CMV-LacZ, Ad/GT-LacZ, Ad/GT-Bax, and Ad/PKG-GV16 were constructed as described previously (19, 20). Ad/CMV-GFP was provided by Dr. T. J. Liu (M. D. Anderson Cancer Center, Houston, TX). Ad/hTERT-LacZ and Ad/hTERT-GV16 were constructed by replacing the CMV promoter with a 200-bp hTERT core promoter region.

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3 The abbreviations used are: CMV, cytomegalovirus; TERT, telomerase reverse transcriptase; hTERT, human TERT; NHPB, normal human fibroblast; NHBE, normal human bronchial epithelial; MOI, multiplicity of infection; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt; AST, aspartate transaminase; ALT, alanine transaminase; PGK, 3-phosphoglycerate.

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85% of human cancers but is quiescent in most normal somatic cells. To test the feasibility of using the hTERT promoter to induce tumor-specific transgene expression in cancer gene therapy, we constructed an adenoviral vector expressing a LacZ reporter gene driven by the hTERT core promoter and evaluated its activity in vitro and in vivo. The hTERT promoter could drive high-level expression of LacZ in tumor cells but not in normal cells and normal mouse tissues. Using a binary adenoviral system that can induce Bax gene expression, we showed that induction of the Bax gene expression via the hTERT promoter elicited tumor-specific apoptosis in vitro, suppressed tumor growth in nude mice, and prevented the toxicity of the Bax gene in vitro and in vivo. Thus, the hTERT promoter is apparently a strong and tumor-selective promoter with potential application in targeted cancer gene therapy.
**Analysis of in Vitro Gene Expression.** Human lung cancer cell lines A549 and H1299 and cervical cancer cell line HeLa were originally obtained from American Type Culture Collection and maintained in our laboratory (M. D. Anderson Cancer Center). Human colon cancer cell lines DLD1 and LoVo were obtained from Dr. T. Fujiwara (Okayama University, Okayama, Japan). NHFB cells and NHBE cells were purchased from Clonetics (San Diego, CA) and cultured in media recommended by the manufacturer. Cells were plated 1 day prior to infection at densities of $1 \times 10^5$ cells/well in a 24-well plate. Cells were then infected with adenoviral vectors at a MOI of 1000 viral particles/cell. Twenty-four h after infection, cells were either stained with X-Gal to visualize β-galactosidase expression or harvested for biochemical analysis of β-galactosidase activity.

**Biochemical Analysis.** Cultured cells were lysed or tissues from BALB/c mice were homogenized in β-galactosidase assay buffer. Cell or tissue debris was then removed by microcentrifugation. Protein concentrations were determined using a kit from Pierce according to the manufacturer’s instructions. β-galactosidase activities were determined using a luminometer and a Galacto-Light Chemiluminescent Assay kit from Tropix, Inc. (Bedford, MA).

**Cell Viability Assay.** Cells were plated on 96-well plates at $1 \times 10^4$ per well 1 day prior to virus infection. Cells were then infected with adenoviral vectors at a total MOI of 1500 viral particles/cell. Cells were divided into four groups according to the viral vector system given: Ad/CMV-GFP + Ad/PGK-GV16, Ad/GT-Bax + Ad/CMV-GFP, Ad/GT-Bax + Ad/hTERT-GV16, or Ad/GT-Bax + Ad/PGK-GV16. 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 (20). PBS was used for mock infection. The cell viability was determined by XTT assay using a Cell Proliferation Kit II (Roche Diagnostics) according to the manufacturer’s protocol. In each treatment group, quadruplicate wells were measured for cell viability.

**Aptoptosis Analysis by Flow Cytometry.** Cells were plated at densities of $1 \times 10^5/100$-mm plate 1 day prior to infection. The cells were then infected with recombinant adenoviral vectors at a MOI of 1500 viral particles/cell. Forty-eight h later, both adherent and floating cells were harvested by trypsinization, washed with PBS, and fixed in 70% ethanol overnight. Cells were then stained with propidium iodide for analysis of DNA content. Apoptotic cells were quantified by flow cytometric analysis performed in the Flow Cytometry Core Laboratory at our institution (M. D. Anderson Cancer Center).

**Animal Experiments.** All of the mice were cared for according to 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. In vivo infusion of adenoviral vectors into and subsequent tissue removal from BALB/c mice were done as described previously (19). In the s.c. tumor model, $5 \times 10^5$ H1299 cells were inoculated s.c. into the dorsal flank of 6- to 8-week-old nude mice (Harlan Sprague Dawley, Indianapolis) to establish tumors. After tumors reached ~5 mm in diameter, mice were given three sequential intratumoral injections of $9 \times 10^9$ viral particles in a volume of 100 μl per dose. Tumor sizes were measured three times a week. Tumor volumes were calculated using the formula $a \times b^2 \times 0.5$, where $a$ and $b$ represent the larger and smaller diameters, respectively.

**Histochemistry Study.** For H&E staining, sectioned tissues or tumors were processed by the Histology Laboratory in the Department of Veterinary Medicine and Surgery at our institution. For X-Gal staining, 8-μm-thick frozen sections were fixed with 50% ethanol and 50% methanol for 20 min at −20°C. The fixed sections were then stained with a solution, containing 5 mM K$_4$Fe(CN)$_6$, 5 mM K$_3$Fe(CN)$_6$, 2 mM MgCl$_2$, and 1 mg/ml X-Gal, at 37°C overnight and were finally counterstained with Nuclear Fast Red (Sigma).

**Analysis of Serum AST and ALT.** Blood was drawn from the tail vein of mice 48 h after adenovirus infusion. The levels of serum AST and ALT were measured as described previously (21).

**Statistical Analysis.** Differences among the treatment groups were assessed by ANOVA using statistical software (StatSoft, Tulsa, OK). $P \leq 0.05$ was considered significant.

**Results**

**Tumor-specific Transgene Expression Driven by the hTERT Promoter in Vitro.** To assess transgene expression from the hTERT promoter in various cells, we first constructed an adenoviral vector expressing the LacZ gene driven by a 378 bp hTERT core promoter (16). The hTERT promoter activity was assessed in cultured human lung cancer cell lines (H1299 and A549), colon cancer cells (DLD1 and LoVo), cervical cancer cells (HeLa), NHFB cells, and NHBE cells by infecting the cells at a MOI of 1000 viral particles. Expression of bacterial β-galactosidase was then analyzed 24 h after infection by either X-Gal staining or enzyme assay as described in “Materials and Methods.” In all of the cancer cell lines tested, both the CMV and hTERT promoters drove strong β-galactosidase expression as evidenced by X-Gal staining, whereas in the two normal cell lines, only infection with Ad/CMV-LacZ produced high levels of transgene expression (nearly 100%). Infection of the normal cells at the same MOI with Ad/hTERT-LacZ resulted in very few LacZ-positive cells (Fig. 1A). CMV and hTERT promoter activity differed by only 2- to 10-fold in cancer cells compared with more than a 500-fold difference in normal cells (Fig. 1B). In all of the cells tested, hTERT promoter activity was significantly higher in cancer cells than in normal cells ($P \leq 0.05$). These results together demonstrated that the hTERT promoter was highly active in a variety of cancer cell lines but not in normal cells, thus suggesting that the hTERT promoter is both strong
TUMOR-SPECIFIC TRANSGENE EXPRESSION FROM hTERT PROMOTER

**Transcriptional Activity of the hTERT Promoter in Vivo.** To investigate the levels of transgene expression induced by the hTERT promoter in vivo, we infused 6 × 10⁹ particles of Ad/hTERT-LacZ, Ad/CMV-LacZ, or Ad/CMV-GFP into BALB/c mice via the tail vein. All of the mice were killed 2 days after vector or PBS infusion; and the liver, spleen, heart, lung, kidney, intestine, ovary, and brain were removed from each for histochemical staining and biochemical analyses of bacterial β-galactosidase expression. High levels of β-galactosidase activity were detected in the livers and spleens of mice treated with Ad/CMV-LacZ. The enzyme activities in other organs of mice treated with Ad/CMV-LacZ were the same as in the background controls. In contrast, the enzyme activities in the livers, spleens, and other organs of mice treated with Ad/hTERT-LacZ were all within the ranges seen in background controls, i.e., PBS- and Ad/CMV-GFP-treated mice (Fig. 2, A and B). The failure of the hTERT promoter to drive detectable LacZ expression in adult mouse tissues was not attributable to the inability of the hTERT promoter to use the mouse transcriptional machinery, inasmuch as a high level of transgene expression was detected in a mouse lung carcinoma cell line (M109) after infection with Ad/hTERT-LacZ (data not shown). It is noteworthy that the promoter region of the mouse TERT gene was also recently cloned; the E-box and two Sp1 binding sites in the core promoter region, which were believed to be critical for their high expression in cancer cells, were found to be conserved between hTERT and mouse TERT (22). These data together suggest that the hTERT promoter can be used to prevent transgene expression in normal liver and spleen cells and to minimize the liver and spleen toxicity of a therapeutic gene after its systemic delivery.

**hTERT Promoter-driven Bax Gene Expression Specifically Suppresses Tumor Cells in Vitro.** We have recently developed a binary adenoviral vector system that enables us to overcome the difficulties in constructing adenoviral vectors expressing high levels of the strongly apoptotic Bax gene (20, 21). In brief, the system contains two adenoviral vectors. One of these vectors contains a human Bax cDNA under the control of a minimal synthetic promoter comprising five Gal-4 binding sites and a TATA box, which is dormant in 293 packaging cells, thus avoiding the toxic effects of the Bax gene on the 293 cells and allowing vector (Ad/GT-Bax) production. The expression of the Bax gene can be induced by coinfected the Ad/GT-Bax virus with the second adenoviral vector in the binary system (Ad/PGK-GV16). Ad/PGK-GV16 contains a synthetic transactivator, consisting of a fusion protein comprised of a Gal-4 DNA-binding domain and a VP 16 activation domain under the control of a constitutively active PGK promoter, a housekeeping gene promoter from the mouse 3-phosphoglycerate kinase gene. Previously, it was shown that administration of this binary vector system to cancer cells elicited extensive apoptosis in vitro and suppressed tumor growth in vivo (20, 21). However, systemic administration of the vector system also resulted in massive apoptosis in the liver, which suggested that overexpression of Bax gene is toxic to normal cells (20).

To test whether the hTERT promoter can be used to negate the toxic effects of the Bax gene on normal cells while preserving its antitumor activity, we constructed a recombinant adenoviral vector (Ad/hTERT-GV16) by replacing the PGK promoter in Ad/PGK-GV16 with the hTERT promoter. The effects of the Bax gene on normal and tumor cells when induced by the hTERT promoter compared with the effects when induced by the PGK promoter were then tested using the binary adenoviral vector system (Fig. 3A). Human lung cancer lines H1299 and A549, NHBE cells, and NHFB cells were treated with PBS, Ad/CMV-GFP, Ad/CMV-GFP, Ad/GT-Bax, Ad/CMV-GFP, Ad/GT-Bax, or Ad/PGK-GV16. The cells were harvested 48 h after the treatment and subjected to fluorescence-activated cell sorter analysis to determine the fraction of apoptotic cells by quantifying the sub-G₁ population. Induction of apoptosis in H1299 and A549 cells was comparable after infection with either Ad/GT-Bax + Ad/hTERT-GV16 or Ad/GT-Bax + Ad/PGK-GV16, which suggests that the hTERT promoter is as strong as the PGK promoter in inducing Bax gene expression and apoptosis in tumor cells. In the two normal cell lines (NHBE and NHFB), however, treatment with Ad/GT-Bax + Ad/PGK-GV16 elicited substantial apoptosis as well, whereas treatment with Ad/GT-Bax + Ad/hTERT-GV16 elicited no obvious apoptosis. These results demonstrated that the hTERT promoter can be used to drive tumor-specific proapoptotic gene expression and apoptosis induction while negating the toxicity of a proapoptotic gene to normal cells.

To obtain further evidence that the hTERT promoter could drive specific expression of the Bax gene in tumor cells but not in normal cells, we used the XTT assay to compare cell viability after treatment with either Ad/GT-Bax + Ad/PGK-GV16 or Ad/GT-Bax + Ad/hTERT-GV16. Treatment with either binary vector had comparable cell-killing effects on H1299 and A549 cells. However, in NHBE and NHFB cells, treatment with Ad/GT-Bax + Ad/PGK-GV16 also caused dramatic cell loss, whereas treatment with Ad/GT-Bax + Ad/hTERT-GV16 had only a minimal effect on cell viability (Fig. 3B). The results were further supported by Western blot analysis. Both hTERT and PGK promoters induced strong Bax expression in A549 and liver (22). The results are from one of five similarly treated mice per group. Values, means + SD for five mice per group.
cells. In comparison, PGK but not hTERT promoter induced strong Bax expression in NHFB cells (Fig. 3C).

Bax Gene Expression Driven by the hTERT Promoter Suppresses Tumor Growth in Vivo. To evaluate the possibility of using the hTERT promoter for in vivo Bax gene therapy, we established H1299 tumors s.c. in nude mice and treated the tumors with the Bax gene expression of which was driven by the hTERT or PGK promoter. After three sequential intratumoral injections of adenoviral vectors, tumor size changes were monitored for 3 weeks. Treatment with Ad/GT-Bax + Ad/hTERT-GV16 or Ad/GT-Bax + Ad/PGK-GV16 resulted in the same levels of tumor-growth suppression that were significantly different from treatments with PBS, Ad/E1B- or Ad/GT-LacZ + Ad/hTERT-GV16 groups (P ≤ 0.001; Fig. 4). These results demonstrated that the hTERT promoter can effectively drive transgene expression in tumors in vivo.

hTERT Promoter Prevents Liver Toxicity of the Bax Gene. To test whether the hTERT promoter can be used to prevent the toxicity of Bax gene expression in the liver after systemic gene delivery, adult BALB/c or nude mice were infused via the tail vein with PBS, Ad/GT-Bax + Ad/CMV-GFP, Ad/GT-Bax + Ad/hTERT-GV16, or Ad/GT-Bax + Ad/PGK-GV16 at a total dose of 6 × 10^10 viral particles/mouse. Mice were killed 24 h after treatment, and their livers were harvested for histological examination. As shown in Fig. 5A, panel d, the majority of hepatocytes have undergone apoptosis after PGK promoter-induced Bax expression. In comparison, when Ad/GT-Bax + Ad/hTERT-GV16 was infused, very few apoptotic hepatocytes were observed (Fig. 5A, panel c). To further document the liver toxicity by the Bax gene treatment, blood samples were collected 48 h after i.v. virus injection, and the serum levels of liver AST and ALT were determined (Fig. 5B). Although treatments with PBS, Ad/GT-LacZ + Ad/PGK-GV16, or Ad/GT-Bax + Ad/hTERT-GV16 resulted in the same serum AST and ALT levels, the treatment with Ad/GT-Bax + Ad/PGK-GV16 resulted in >16- and 41-fold increases in AST and ALT levels, respectively (P ≤ 0.0001). Together, these results suggest that hTERT promoter can be used to prevent the liver toxicity of proapoptotic genes.

Discussion

Targeted expression of therapeutic genes can be accomplished at several levels, including direct injection into a target site, vector targeting, and tissue-specific gene expression (23). Whereas intratumoral injection is appropriate for the treatment of unselectable primary tumors, systemic gene delivery may be necessary for the treatment of metastasis. However, systemic delivery of the Bax gene induces substantial liver toxicity (20), which suggests that tumor-specific transgene expression may be essential for the systemic delivery of such genes in the clinic.

Targeted transgene expression may also be achieved by receptor-mediated gene targeting. This method has been exploited primarily for
nonviral gene delivery through the use of molecular conjugates and protein/DNA complexes. A variety of ligands and receptors have been used for the delivery of genes to specific tissues or cells, such as targeting hepatocytes via asialoglycoprotein receptor (24) and targeting macrophages via mannose receptors (25). Targeted gene delivery via viral vectors has also been tested by conjugating preformed vector particles with antibodies, and by manipulating the genes encoding the viral capsid or coat proteins so as to produce viral particles with modified surface proteins. Modification of adeno viral surface proteins to alter the natural tropism of adenovirus and to permit gene transfer into specific cell types has been reported (26, 27). However, no tumor-specific gene delivery system with broad application has yet been developed.

We have shown here that the hTERT promoter has high transcriptional activity in a variety of human cancer cell lines and can be used to prevent the toxic effects of the Bax gene in vitro and in vivo without compromising the antitumor activity of the gene. Thus, hTERT should be useful for targeting the pharmaceutical effects of a therapeutic gene to cancer cells. Indeed, the fact that telomerase is active in various tumor types and in >85% of all primary tumors suggests that the hTERT promoter will find broad applications in cancer gene therapy.

It is intriguing that the relatively short hTERT core promoter acts so remarkably differently in tumor versus normal cells, although the mechanisms responsible are still not quite understood. However, the widespread expression of hTERT in heterogeneous tumors suggests that general transcriptional factors, rather than any specific transcriptional factors, are responsible for the up-regulation of hTERT in tumor cells. Several reports have shown that c-Myc, an E-Box binding protein, can directly activate hTERT transcription (16, 17, 28, 29). On the other hand, although hTERT expression can be activated by c-Myc in telomerase-competent cells, the levels are not as high as those seen in tumor cells, which strongly suggests that active repression mechanisms may exist in normal cells.

One of the major concerns about the use of the hTERT promoter to drive expression of proapoptotic or cytotoxic genes is its potential toxicity to stem cells. However, evidence suggests that hTERT may not be active in quiescent or G0 stem cells (30). In fact, a study on telomerase activities in bone marrow cells showed that primitive stem cells and their mature derivatives had lower basal levels of telomerase activity, whereas the early progenitors had higher levels (31), which suggested that toxic effects, if any, on stem cells arising from the hTERT promoter may be transient. Furthermore, the toxic effects on stem cells can also be prevented by selecting vectors that poorly transduce stem cells. For example, adeno viral vectors infect stem cells poorly. A very high dose of an adeno viral vector and prolonged cell-vector contact are required to infect stem cells, and, even at high doses of adeno viral vector, only a limited percentage of stem cells will be infected (32). Nevertheless, it will be important to investigate the long-term toxic effects of proapoptotic or cytotoxic genes the expression of which is driven by the hTERT promoter delivered by a particular vector system.

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Tumor-specific Transgene Expression from the Human Telomerase Reverse Transcriptase Promoter Enables Targeting of the Therapeutic Effects of the Bax Gene to Cancers

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