Low-Dose Etoposide Enhances Telomerase-Dependent Adenovirus-Mediated Cytosine Deaminase Gene Therapy through Augmentation of Adenoviral Infection and Transgene Expression in a Syngeneic Bladder Tumor Model

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Abstract

The human telomerase reverse transcriptase (hTERT) promoter can selectively drive transgene expression in many telomerase-positive human cancer cells. Here we evaluated combination therapy of adenoviral vector Ad-hTERT-CD encoding E. coli cytosine deaminase (CD) driven by the hTERT promoter and low-dose etoposide (0.1 μg/ml) for treating bladder cancer. Ad-hTERT-CD conferred sensitivity to 5-fluorocytosine (5-FC) in bladder cancer cells, which could be enhanced by etoposide treatment, but not in normal cells. Such effect was correlated with up-regulation of hypoxia-inducible factor (HIF)-1α expression. By contrast, etoposide activated p53 and down-regulated hTERT promoter activity in normal cells. Etoposide also increased adenoviral infection via enhancement of coxsackie-adenovirus receptor expression on bladder cancer and normal cells. Combination index analysis revealed that combined therapy of Ad-hTERT-CD (105 plaque-forming units)/5-FC (200 mg/kg) with etoposide (2 mg/kg) synergistically suppressed tumor growth and prolonged survival in mice bearing syngeneic MBT-2 bladder tumors. This combination therapy regimen induced complete tumor regression and generated antitumor immunity in 75% of tumor-bearing mice. Furthermore, increased infiltrating CD4+ and CD8+ T cells and necrosis within tumors were found in mice receiving combination therapy of Ad-hTERT-CD and etoposide compared with those treated with either treatment alone. Thus, the potential high therapeutic index of the combination therapy may be an appealing therapeutic intervention for bladder cancer. Furthermore, because a majority of human tumors exhibit high telomerase activity, adenovirus-mediated CD gene therapy driven by the hTERT promoter in combination with low-dose etoposide may be applicable to a broad spectrum of cancers. (Cancer Res 2006; 66(20): 9957–66)

Introduction

Telomerase, a ribonucleoprotein enzyme involved in the synthesis and maintenance of telomeric repeats in the ends of chromosomes, is expressed in immortalized cell lines and in ~90% of human malignancies, but not in most adult somatic tissues (1). The human telomerase reverse transcriptase (hTERT) is the catalytic subunit for human telomerase and its expression is highly associated with telomerase activity (2–4). Telomerase activity and hTERT mRNA expression are detected in most, if not all, cancers including bladder cancer (5–8). By contrast, normal tissues, including those adjacent to cancer, display no or very low levels of telomerase activity. Moreover, the levels of hTERT expression are significantly associated with bladder tumor grade and stage (9). Therefore, hTERT may serve as a good target for gene therapy of bladder cancer and also be useful for targeted transgene expression in human and murine cancers (10–12). The use of hTERT promoter-driven vector system is able to restrict transgene expression to telomerase-positive tumors. Because the vast majority of human bladder cancers express telomerase activity, the hTERT promoter may be exploited for bladder tumor–specific transgene expression in gene therapy of bladder cancer.

Etoposide causes DNA strand breaks and induces apoptosis in a variety of tumor cells. Etoposide up-regulates telomerase activity in human pancreatic cancer cells (13). Furthermore, etoposide at clinically acceptable dosages suppresses humoral and cellular immune responses to adenoviral vectors, thereby enhancing intratumoral transgene expression (14). We hypothesized that low-dose etoposide can increase the infection efficacy of adenoviral vector encoding cytosine deaminase (CD) under the transcriptional control of the hTERT promoter and enhance CD gene expression through up-regulation of the hTERT promoter activity in telomerase-positive bladder cancer cells. Here we show that low-dose etoposide increased the hTERT promoter activity through up-regulation of hypoxia-inducible factor (HIF)-1α expression and enhanced adenoviral infection via up-regulation of coxsackie-adenovirus receptor (CAR) in bladder cancer cells. Therefore, the combination of etoposide and adenovirus-mediated CD gene therapy driven by the hTERT promoter may have potential applications for the treatment of bladder cancer or other telomerase-positive malignancies.

Materials and Methods

Cells and mice. Normal murine fibroblasts were isolated from adult C3H/HeN mice using standard methods. Seven human cell lines (J82, T24, TSGH-8301, TCC-SUP, and HT-1376 bladder cancer cells as well as WI-38 fetal lung fibroblast and HepG2 hepatocellular carcinoma cells) and four murine cells (MBT-2 bladder transitional cell carcinoma, Li2 Lewis lung carcinoma, NIH 3T3 embryonic fibroblast, and adult fibroblast) were...
cultured in DMEM supplemented with 10% fetal bovine serum or bovine serum (for NIH 3T3 cells), 2 mmol/L l-glutamine, and 50 μg/mL gentamicin. Male, 6- to 8-week-old C3H/HeN mice were obtained from the Laboratory Animal Center of the National Cheng Kung University. The animals were maintained in specific pathogen-free animal care facility under isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan and was approved by the Laboratory Animal Care and Use Committee of the National Cheng Kung University.

**Analyses of hTERT promoter activity and p53 and HIF-1 transcriptional activities.** To quantitatively determine the hTERT promoter activity, cells grown in 24-well plates were cotransfected with 1 μg of pGL3-hTERT (15), a luciferase reporter plasmid driven by the hTERT promoter, and 0.5 μg of pTRE LacZ, a β-galactosidase (β-gal) reporter vector derived from pTRE2ZEGFP (Clontech, Palo Alto, CA), with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). In some experiments, at 6 hours posttransfection, cells were treated with various doses of etoposide (Bristol-Myers Squibb, Princeton, NJ). Cell lysates were harvested at 48 hours posttransfection for measuring luciferase activities as previously described (16). Cells were also cotransfected with 1 μg of p53-Luc reporter plasmid and 0.5 μg of pTRE LacZ for assessing the transcriptional activity of p53 (16). A DNA fragment containing six tandemly repeated sequences of hypoxia response element (HRE) from the human lactate dehydrogenase A gene (17) just upstream of the cytomegalovirus (CMV) mini promoter derived from pTRE vector (Clontech) was placed upstream of the luciferase reporter gene in a pGL3-Basic vector (Promega, Madison, WI) to generate pHRECMVmini-Luc. Previously, we found that the HIF-1 consensus binding site at −184 relative to the transcription start site of the hTERT promoter was required for induction of the hTERT promoter activity by hypoxia, as determined by deletion analysis and site-directed mutagenesis.6 We therefore used pGL3-184-mhTERT, which carries TTT rather than CAC in the putative HIF-1 binding site located at −184 bp upstream of the transcriptional start site of the hTERT gene, to verify the involvement of HIF-1 in up-regulating hTERT promoter activity by etoposide. The HIF-1 transcriptional activity of etoposide-treated cells was examined using the same protocol as that for assessing p53 transcriptional activity except that pHRECMVmini-Luc or pGL3-184-mhTERT rather than p53-Luc was used. To further confirm the role of HIF-1 on etoposide-induced up-regulation of the hTERT promoter activity, pSUPER vector (ref. 18; kindly provided by Dr. R. Agami, The Netherlands Cancer Institute, Amsterdam, the Netherlands) was used for stable expression of hTERT in various cell lines.

**Immunoblot analysis.** MBT-2 cells were cultured with or without etoposide for 6 hours and total cell lysates were harvested for detection of HIF-1α expression by immunoblot analysis with rabbit polyclonal antibody against HIF-1α (1:1,000; ab16535, Abcam, Cambridge, United Kingdom). Total lysates from LL2 and HepG2 cells treated with CoCl2 (200 μmol/L) for 6 hours served as the positive control. The blot reprobed with anti-α-tubulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) served as the internal control.

**Flow cytometric analysis of CAR and integrin expressions on bladder cancer cells after etoposide treatment.** Cells that had been treated with etoposide (0.1 μg/mL) for 6 hours were incubated on ice with anti-CAR (1:200; RmcB, American Type Culture Collection, Manassas, VA), anti-α5β1 (1:50; 23C6, PharMingen, San Diego, CA), or anti-α6β4 (1:200; P1F6, Chemicon, Temecula, CA) monoclonal antibodies for 1 hour followed by incubation for 30 minutes on ice with FITC-conjugated goat anti-mouse antibody, isotype immunoglobulin G1 (IgG1) was used as a matched control. These samples were analyzed with a FACS Calibur flow cytometer (Becton Dickinson, San Jose, CA).

**Animal studies.** MBT-2 cells (2 × 10⁵) were inoculated s.c. into the dorsal flank of C3H/HeN mice at day 0. At day 10, visible and palpable nodules developed at all injection sites ranging from 100 to 300 mm³, with mean tumor volume of 197 ± 3 mm³. Five groups of seven to nine mice were injected i.t. at different stages and injection sites with 10⁵ plaque-forming units (pfu) of Ad-hTERT-CD or Ad-LacZ in 100 μL of PBS, or with PBS daily at days 11, 12, and 13. One group receiving Ad-hTERT-CD and another group receiving Ad-LacZ were further treated daily with 5-FC (500 mg/kg) i.p. for 14 consecutive days starting on day 12. The remaining three groups received PBS instead. In a separate experiment, four groups of eight tumor-bearing mice were treated with Ad-hTERT-CD (10⁴ or 10⁵ pfu) or PBS daily at days 11, 12, and 13 followed by daily treatment with 5-FC (200 mg/kg) for 7 consecutive days starting on day 12. Additionally, all groups, except one Ad-hTERT-CD (10⁵ pfu) group receiving PBS, were also treated i.p. with etoposide at 2 mg/kg/d, which was defined as a low dose (14), for 8 consecutive days starting on day 11. Tumor volumes were measured as previously described (16). The animals were sacrificed when their primary tumor reached 10% of the body weight.

**Histologic and immunohistochemical analyses.** To analyze necrosis and cell infiltrates within tumors, tumor-bearing mice treated with different regimens as described above were sacrificed at day 21 and three tumors from each group were excised and fixed in 10% formalin overnight. The tissues were dehydrated and embedded in paraffin and sectioned at 5 μm for H&E or immunohistochemical staining. To detect CD4+ and CD8+ T cells that infiltrated tumors, tumor sections were incubated with rat anti-mouse CD4 (L3T4; GK1.5, PharMingen) or rat anti-mouse CD8α (Ly-2; 53-6.7, PharMingen) antibody and subsequently with biotinylated secondary antibodies and the avidin-biotin-peroxidase complex (DAKO, Carpinteria, CA). Reactions were developed with amino ethyl carbazole (Zymed, South San Francisco, CA) as the chromogenic substrate.

**Statistical analysis.** The survival analysis was done using the Kaplan-Meier survival curve and the log-rank test. Other statistical differences were assessed with Student’s t test. P < 0.05 is regarded statistically significant. Using the CalcuSyn software program (Biosoft, Cambridge, United Kingdom) according to the combination index method of Chou and Talalay (22), significant synergism was determined.

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Results

Ad-hTERT-CD/5-FC exerted cytotoxic effect in vitro and antitumor effect in vivo against bladder cancer cells. We first assessed the hTERT promoter activity and cell survival following Ad-hTERT-CD/5-FC treatment in various cells using a luciferase reporter assay (Fig. 1A). MBT-2 cells exhibited much higher hTERT promoter activities compared with mouse fibroblasts and NIH 3T3 cells, indicating that the hTERT promoter was significantly active in murine bladder cancer cells but relatively quiescent in normal fibroblasts. Notably, as NIH 3T3 fibroblasts were derived from spontaneously immortalized mouse embryo culture, they expressed detectable levels of telomerase activity (23). In the presence of 500 μmol/L 5-FC, Ad-hTERT-CD at an MOI of 1 exerted potent cytotoxic effects on MBT-2 cells, whereas cell survival was not affected in NIH 3T3 cells or normal fibroblasts following Ad-hTERT-CD treatment. Regarding human cells, the hTERT promoter activity varied in different human bladder cancer cell lines, whereas it was not detectable in WI-38 fibroblasts. Furthermore, the cytotoxicity of Ad-hTERT-CD/5-FC was largely proportional to the level of hTERT promoter activity.

![Image](https://example.com/image1.png)

**Figure 1.** Ad-hTERT-CD/5-FC exerted cytotoxic effect, which was proportional to the hTERT promoter activity, and antitumor effect on bladder cancer cells. A, various cell lines were assessed for their hTERT promoter activities by a luciferase reporter assay. Cells were infected with Ad-hTERT-CD at an MOI of 1 in the presence of 500 μmol/L 5-FC, and their survival was determined by the WST-1 assay and expressed as the percentage of surviving cells relative to that in the mock-infected cells without 5-FC treatment. B, HT-1376 and WI-38 cells were infected with indicated doses of Ad-hTERT-CD and 5-FC, and their survival was determined. Columns, mean of three (A) or four (B) determinations, which were consistent in two separate experiments; bars, SD. MBT-2 tumor-bearing C3H/HeN mice were treated i.t. with 107 pfu of Ad-hTERT-CD or Ad-LacZ, or with PBS at days 11, 12, and 13, followed by 5-FC (500 mg/kg) or PBS treatment for 14 consecutive days starting on day 12. C, columns, mean tumor volume; bars, SD (P = 0.0002, Ad-hTERT-CD/5-FC versus PBS). D, Kaplan-Meier survival curves (P = 0.0001, Ad-hTERT-CD/5-FC versus PBS).
Low-dose etoposide enhanced the hTERT promoter activity through up-regulation of HIF-1α expression in bladder cancer cells. In MBT-2 cells, etoposide at doses ranging from 0.02 to 0.5 µg/mL enhanced the hTERT promoter activity, with an optimal concentration of 0.1 µg/mL (Fig. 2A). Because the IC₅₀ of etoposide in MBT-2 cells is >100 µmol/L (25), etoposide at a dose of 0.1 µg/mL (1.7 µmol/L), which was regarded as a low dose, was chosen for subsequent in vitro experiments. Notably, etoposide treatment had no effect on cell survival (Fig. 2A). Etoposide also increased the hTERT promoter activity in human bladder cancer cells displaying various degrees of promoter activity (Fig. 2B). The hTERT promoter activity was enhanced in MBT-2 cells but decreased in NIH 3T3 cells when treated with etoposide (Fig. 2C). These results suggest that following etoposide treatment, the hTERT promoter was significantly active in bladder cancer cells but relatively quiescent in immortalized cells. As etoposide can activate p53 and thereby down-regulate hTERT gene expression (26), we also assessed the effects of p53 status on etoposide-induced up-regulation of the hTERT promoter activity (Fig. 2C). Etoposide increased the hTERT promoter activity ~1.5-fold in MBT-2 cells that harbor mutant p53. By contrast, etoposide decreased the hTERT promoter activity in NIH 3T3 cells, which was associated with enhanced p53 transcriptional activity. Physiologic stress conditions, such as hypoxia, play a role in developing drug resistance in solid tumors. Cancer cells under hypoxic conditions become resistant to multiple drugs, including etoposide. The presence of HIF-1 binding sites on the hTERT promoter prompted us to examine whether HIF-1 was involved in etoposide-induced up-regulation of the hTERT promoter activity in MBT-2 cells. The expression of HIF-1 was significantly up-regulated in etoposide-treated MBT-2 cells, as measured by a hypoxia-responsive luciferase reporter assay (Fig. 3A) and verified by immunoblot analysis (Fig. 3B). Interestingly, etoposide induced HIF-1 transcriptional activity in MBT-2 but not in NIH 3T3 cells (Fig. 3A). We also used pGL3-184m-hTERT, which carries mutations in the putative HIF-1 binding site of the hTERT gene, to show that mutation of the HIF-1 binding site abolished the activation of the hTERT promoter by etoposide in MBT-2 cells (Fig. 3C). Notably, mutant promoter decreased the basal transcriptional activity by ~30% as compared with the wild-type promoter (Fig. 2A). To further confirm our results, we also determined whether knock-down of HIF-1α expression by shRNA suppressed etoposide-induced up-regulation of the hTERT promoter activity (Fig. 3D). Whereas control shRNA did not affect the hTERT promoter activity in our reporter assay, HIF-1α shRNA abrogated etoposide-induced enhancement of the hTERT promoter activity. Taken together, these results suggest that etoposide-induced activation of the hTERT promoter can be mediated via the transcription factor HIF-1 binding to the HRE in the hTERT promoter in bladder cancer cells.

Low-dose etoposide enhanced adenovirus infection through up-regulation of CAR, but not of αvβ3 or αvβ5 integrins, on bladder cancer cells. Efficient adenovirus infection requires CAR and αvβ integrins for attachment and internalization to enter host cells.
cells. To study the effects of etoposide on adenovirus infectivity in bladder cancer cells, expressions of CAR as well as \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) integrins were detected by flow cytometric analysis. As shown in Fig. 4A, J82 cells expressed moderate levels whereas TCC-SUP cells expressed high levels of CAR. However, both cells expressed little \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) integrin expressions. Furthermore, etoposide enhanced CAR expression on both cells but had little effects on \(\alpha_v\beta_3\) and \(\alpha_v\beta_5\) integrin expressions. The increase of cell-surface CAR may have led to an increase in transgene expression and subsequent protein production. We also confirmed that the increased CAR level allowed increased adenovirus entry into cells. In J82, TCC-SUP, MBT-2, and NIH 3T3 cells, etoposide treatment enhanced \(\beta\)-gal activities in Ad-LacZ-infected cells (Fig. 4B), suggesting that enhanced efficiency of adenoviral transgene expression by etoposide treatment may have been attributed, in part, to the up-regulation of CAR expression. Notably, not only bladder cancer cells but also NIH 3T3 fibroblasts became more susceptible to adenoviral infection after etoposide treatment.

**Low-dose etoposide enhanced cytotoxic activity of Ad-hTERT-CD/5-FC in various bladder cancer cells.** The effect of low-dose etoposide on the cytotoxicity of Ad-hTERT-CD/5-FC was assessed in human and murine cell lines. Cell survival was decreased as the doses of Ad-hTERT-CD and 5-FC were increased in human TSGH-8301, T24, J82, and TCC-SUP bladder cancer cells (Fig. 5A). The degree of cytotoxicity seemed to be a function of adenoviral dose and 5-FC concentration. Remarkably, the cytotoxic effects were further enhanced when these cells were concomitantly treated with etoposide. In MBT-2 cells, there was a good dose-response relationship between Ad-hTERT-CD/5-FC doses and cytotoxicity (Fig. 5B). However, Ad-hTERT-CD/5-FC had only negligible effects on cell survival in NIH 3T3 cells regardless of treatment with etoposide (Fig. 5B). Results with higher doses of Ad-hTERT-CD or 5-FC were the exception, displaying a small degree of cytotoxicity in the absence of etoposide. Nevertheless, etoposide treatment suppressed the cytotoxic effect induced by Ad-hTERT-CD/5-FC. Notably, cell survival remained unaffected after treatment with Ad-hTERT-CD, 5-FC, etoposide, or Ad-LacZ/5-FC alone (data not shown). Collectively, Ad-hTERT-CD conferred sensitivity to 5-FC in human and murine bladder cancer cells, which could be enhanced by etoposide, but not in normal cells. These results suggest that low-dose etoposide may improve the therapeutic index of Ad-hTERT-CD/5-FC for killing telomerase-positive bladder cancer cells.

**Ad-hTERT-CD/5-FC in combination with low-dose etoposide synergistically suppressed tumor growth and prolonged survival in mice bearing syngeneic MBT-2 tumors.** Although Ad-hTERT-CD alone followed by 5-FC treatment slowed tumor growth in MBT-2 tumor-bearing mice (Fig. 1C), the survival rate was only enhanced from 0% to 30% (Fig. 1D). Based on the in vitro data described above, we sought to enhance the therapeutic effect of Ad-hTERT-CD/5-FC by combination with low-dose etoposide. The severe side effects of 5-FC, including hepatotoxicity and bone marrow depression, are concentration dependent and can be elicited by many other agents, such as immunosuppressive or cytotoxic
agents (27). To avoid any possible side effects of the combination therapy, 500 mg/kg 5-FC for 14 days, which was considered the standard treatment regimen (28), was replaced by 200 mg/kg 5-FC for 7 days. As shown in Fig. 6A and B, whereas low-dose etoposide had no antitumor effects, Ad-hTERT-CD/5-FC (10^9 pfu) alone retarded tumor growth (P = 0.0002) and prolonged survival (P = 0.0003) in MBT-2 tumor–bearing mice compared with etoposide treatment alone. Ad-hTERT-CD alone inhibited tumor growth by 22 days, with regrowth of tumors thereafter when 5-FC was no longer administered. Remarkably, Ad-hTERT-CD (10^9 pfu)/5-FC in combination with etoposide synergistically suppressed tumor growth and enhanced survival in tumor-bearing mice compared with either Ad-hTERT-CD/5-FC (P = 0.0002 for tumor volume and P = 0.0003 for survival) or etoposide (P = 0.0001 for tumor volume and P = 0.0001 for survival) treatment alone. The synergistic effect between etoposide and Ad-hTERT-CD/5-FC treatment groups was verified by combination index values (combination index < 1 represents synergistic activity; combination index = 1 represents additive activity). For the combination therapy, the combination index values from tumor volume data for high and low viral doses were 0.03 and 0.09, whereas those from survival time data were 0.17 and 0.24, respectively. Furthermore, there was no significant difference between mice receiving Ad-hTERT-CD (10^9 pfu)/5-FC and those receiving Ad-hTERT-CD (10^8 pfu)/5-FC on tumor volume and survival. Notably, combination of Ad-hTERT-CD/5-FC and etoposide resulted in complete tumor regression in six of eight high-dose Ad-hTERT-CD–treated mice and in four of eight low-dose Ad-hTERT-CD–treated mice without tumor regrowth. Importantly, administration of Ad-hTERT-CD followed by etoposide and 5-FC treatments produced no weight loss or other overt signs of toxicity in mice. In addition, all tumor-free mice that had been rechallenged s.c. with MBT-2 cells (4 x 10^6) on the opposite flank region at day 70 remained tumor-free after 5 months. Therefore, high-dose and low-dose Ad-hTERT-CD/5-FC in combination with low-dose etoposide induced complete tumor regression in 75% and 50% of tumor-bearing mice and generated antitumor immunity, respectively. As shown in Fig. 5C, H&E staining reveals necrosis within tumors from mice treated with Ad-hTERT-CD/5-FC alone. Furthermore, Ad-hTERT-CD/5-FC plus
etoposide induced more tumor necrosis. Immunohistochemical staining shows that CD4+ and CD8+ T cells that infiltrated tumors were significantly increased in the combination treatment group compared with those in the remaining groups. These results suggest that activated immune cells within tumors may have contributed to higher antitumor effects of the combination regimen.

Discussion

The hTERT promoter has been used to drive tumor-specific apoptotic transgene expression in human telomerase-positive cells and suppress xenograft tumor growth in nude mice (10–12) because telomerase levels are low in adult murine tissues and primary cultured cells but high in most tumors cells (29–31). In this study, although Ad-hTERT-CD/5-FC retarded tumor growth initially, tumors recurred in all the treated mice when 5-FC was no longer administered. The transduction efficiency of adenoviral vectors in tumors by i.t. injection may be low (32). Moreover, high MOIs of adenoviral vectors encoding suicide gene may be required to achieve effective killing of tumor cells (33). Therefore, inefficient vector delivery to tumor cells and resistance to suicide gene therapy remained to be resolved.

Figure 5. Low-dose etoposide enhanced cytotoxic activity of Ad-hTERT-CD/5-FC in various bladder cancer cells. Human TSGH-8301, T24, J82 and TCC-SUP bladder cancer cells (A) as well as murine MBT-2 and NIH 3T3 cells (B) were infected with Ad-hTERT-CD at indicated MOI in the presence or absence of etoposide (0.1 µg/mL) for 6 hours. The cells were refed with fresh medium supplemented with indicated concentrations of 5-FC and etoposide (0.1 µg/mL). Cell survival was determined after 3 days by the WST-1 assay and expressed as the percentage of surviving cells relative to that in the mock-infected cells without 5-FC treatment. Columns, mean of four determinations, which were consistent in two separate experiments; bars, SD.
Low-dose etoposide can suppress immune responses to adenoviral vectors and enhance adenovirus-mediated intratumoral transgene expression (14). In this study, we show that low-dose etoposide in conjunction with Ad-hTERT-CD/5-FC significantly enhanced cytotoxic effects against murine bladder cancer cells but failed to induce evident cytotoxicity in NIH 3T3 fibroblasts, even at high MOIs of adenoviral vectors. As NIH 3T3 cells, which were spontaneously immortalized embryonic fibroblasts, expressed detectable levels of telomerase activity (23), they may have expressed CD, albeit to a lesser extent, following high MOIs of Ad-hTERT-CD infection, leading to a small degree of cytotoxic effects. Interestingly, etoposide treatment abolished such effects. Along the same line, our results show that whereas etoposide enhanced the hTERT promoter activity in cancer cells harboring mutant p53, it actually suppressed the promoter activity in NIH 3T3 cells that carry wild-type p53. Moreover, etoposide induced HIF-1 transcriptional activity in MBT-2 but not in NIH 3T3 cells. Our results also show enhanced cytotoxic effects of Ad-hTERT-CD/5-FC by etoposide in a variety of human bladder cancer cells. Possible mechanisms involved in adenoviral transduction seem to be correlated with CAR and integrin expressions on target cells (34–36). It has been shown that adenoviral transduction was enhanced in cisplatin-resistant human laryngeal carcinoma cells, which was associated with increased expressions of $\alpha_v\beta_3$ integrin and CAR (37). To this end, cisplatin and etoposide enhanced CAR expression in two of five human ovarian cancer cell lines tested, which resulted in adenovirus-mediated transgene expression (38). In this study, we show that adenoviral transduction was significantly enhanced in bladder cancer cells after exposure to etoposide, which was associated with increased expression of CAR. These results suggest that enhancement of CAR expression may be one of the mechanisms contributing to the higher cytotoxic and tumoricidal effects of Ad-hTERT-CD/5-FC plus etoposide on MBT-2 cells in vitro and in vivo. In the clinical settings, low efficiency of gene transfer with adenoviral vectors in cancer gene therapy due to low expression of CAR may be improved by administration of etoposide at a clinically acceptable dosage.

In bladder cancer cell lines, responses to Ad-hTERT-CD/5-FC were variable, which could be attributable to the differences not only in the expression level of CD driven by the hTERT promoter but also in the susceptibility to adenoviral infection and sensitivity to 5-FC. It has been reported that whereas both gene transfer
efficiency of adenoviral vector and inherent 5-FU sensitivity significantly contribute to overall 5-FC responses of cells after CD gene transfer, inherent 5-FU sensitivity is a more contributing factor (24). In our in vitro data, cytotoxic effects of Ad-hTERT-CD/5-FC on different cells were largely paralleled with the levels of the hTERT promoter activity. Nevertheless, levels of the hTERT promoter activity were not the only critical factor that determined the cell survival following Ad-hTERT-CD/5-FC treatment. For instance, whereas the hTERT promoter seemed to be less active in HT-1376 cells than in MBT-2 cells (Fig. 1A), they exhibited similar degrees of cytotoxicity induced by Ad-hTERT-CD/5-FC. Based on IC_{50} values, HT-1376 cells were ~170 times more sensitive to 5-FU than MBT-2 cells, which may have decreased their sensitivity threshold to Ad-hTERT-CD/5-FC. Among different bladder cancer cells used in this study, TSGH-8301 cells, which exhibited the lowest hTERT promoter activity but intermediate 5-FU sensitivity, were the most susceptible bladder cancer cell line to adenosiral infection (39). Nevertheless, TSGH-8301 cells were the least sensitive cell line to Ad-hTERT-CD/5-FC-induced cytotoxicity (Fig. 1A). These data suggest that the CD expression level, which was dependent on the hTERT promoter activity, may be an important factor contributing to the cytotoxicity of Ad-hTERT-CD. The cytotoxic effect of Ad-hTERT-CD plus 5-FC was dose dependent in that the cell survival decreased when the doses of adenosiral vectors and/or 5-FC increased. However, in some cells infected with 10 MOI of Ad-hTERT-CD, such as HT-1376 cells (Fig. 1B), 5-FC produced a dose-response decrease in cell survival at doses up to 50 μmol/L. At higher doses, a plateau was reached, suggesting that 5-FU formation may reach a saturation point due to limitation of the CD enzyme via Ad-hTERT-CD gene transfer. Along this line, HT-1376 cells were the least susceptible cells to adenosiral infection (data not shown) and displayed the lowest IC_{50} of 5-FU among the bladder cancer cell lines examined here.

In the present study, the diverse effects of etoposide on the cytotoxicity of Ad-hTERT-CD/5-FC between cancer and normal cells may be attributed, in part, to the level of hTERT promoter activity that was modulated by etoposide. Several transcription factors are involved in the regulation of the hTERT promoter activity. c-Myc and SP1 play as activators, whereas Mad1, p53, and zinc-finger factor MZF2 are repressors (40–44). In this study, our data show that low-dose etoposide enhanced the transcriptional activity of p53, and thereby down-regulated the hTERT promoter activity in NIH 3T3 cells that harbor wild-type p53. Up-regulation of the hTERT promoter activity has been shown to correlate with the activation of HIF-1α transcription factor under hypoxic condition (45, 46). We also show that low-dose etoposide induced HIF-1α expression in MBT-2 cells, leading to enhanced hTERT promoter activity. The HREs in the hTERT promoter were essential for the up-regulation of the promoter by etoposide because the hTERT promoter was not transactivated by etoposide when the HIF-1 binding site was mutated. Furthermore, we found that the basal level of the hTERT promoter activity was decreased by mutations of the HIF-1 binding site (Fig. 3C), which is in accord with previous work (47). Because the HIF-1 consensus binding site (5′-RCGTG-3′) overlaps the E box (CAGCTG) known to bind several transcription factors, such as c-Myc, Max, and Mad (48), HIF-1 may compete with these factors for binding to the E box. Taken together, these results suggest that etoposide-induced activation of the hTERT promoter can be mediated via binding of the transcription factor HIF-1 to the HRE in the hTERT promoter in bladder cancer cells.

In conclusion, low-dose etoposide enhances the antitumor effect of Ad-hTERT-CD/5-FC in bladder tumor-bearing mice by increasing adenoviral infection, up-regulating the hTERT promoter activity, and inducing antitumor immune responses. Notably, we evaluated Ad-hTERT-CD and low-dose etoposide as anticancer agents in the syngeneic MBT-2 bladder tumor model, which resembles more closely the situation in human cancer. In addition, as etoposide activates p53, which down-regulates the hTERT promoter activity in normal cells, it does not confer sensitivity to 5-FC. Thus, combination of low-dose etoposide with Ad-hTERT-CD/5-FC provides therapeutic safety and high efficacy in killing telomerase-positive cancer cells while sparing normal cells. The potential high therapeutic index of Ad-hTERT-CD/5-FC system in combination with etoposide may be an appealing therapeutic intervention for bladder cancer. Because intact urothelium can function as an effective barrier, bladder is suitable for intravesical instillation of adenosiral vectors. In line with this notion, intravesical instillation of adenosiral vectors is safe, feasible, and biologically active in patients with bladder cancer (49). As etoposide is widely used in the clinical settings, it serves as a feasible activator to enhance the therapeutic index of hTERT promoter–driven suicide gene therapy. Because a majority of human tumors exhibit high telomerase activity, adenosivirus-mediated CD gene therapy driven by the hTERT promoter in combination with low-dose etoposide may be applicable to a broad spectrum of cancers.

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References
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