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Advances in Brief

Targeted Expression of Green Fluorescent Protein/Tumor Necrosis Factor-related Apoptosis-inducing Ligand Fusion Protein from Human Telomerase Reverse Transcriptase Promoter Elicits Antitumor Activity without Toxic Effects on Primary Human Hepatocytes[1]

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Abstract

Liver toxicity is the major concern for use of recombinant human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) proteins in treatment of cancers. Here we report that normal human primary hepatocytes (NHPHs) are susceptible to the transduction of the wild-type, full-length coding sequence of the human *TRAIL* gene. To minimize potential toxicity of the *TRAIL* gene, a bicistronic adenoviral vector that expresses the green fluorescent protein/TRAIL fusion protein from the human telomerase reverse transcriptase promoter (designated Ad/gTRAIL) was constructed. *In vitro* and *in vivo* studies have showed that treatment with the adenoviral vector Ad/gTRAIL results in high-level expression of green fluorescent protein/TRAIL in cancer cells but no detectable transgene expression in NHPHs or in normal mouse liver tissues. Furthermore, treatment with Ad/gTRAIL effectively elicited apoptosis in malignant cells but not in NHPHs *in vitro* and suppressed tumor growth and prolonged duration of survival *in vivo*. Thus, with the combined advantages of the *TRAIL* gene and the human telomerase reverse transcriptase target, Ad/gTRAIL can be a potent therapeutic agent for the treatment of cancers.

Introduction

The TRAIL[3] appears to elicit anticancer activity without causing significant toxicity to most normal cells (1). Evidence has shown that repeated i.v. injection of a recombinant and biologically active TRAIL protein induces tumor cell apoptosis, suppresses tumor progression, and improves the survival of animals bearing solid tumors but does not cause detectable toxicity in nonhuman primates (2). However, recent findings that normal human hepatocytes, brain tissue, and certain epithelial cells are susceptible to recombinant TRAIL proteins (3, 4) have raised serious concerns about the potential toxicity of the TRAIL protein if administered systemically. Although some of the observed toxicity could be caused by tagged histidine or leucine in recombinant *TRAIL* proteins (5), further investigation is necessary to document mechanisms of the related toxicity.

We and others recently have showed that direct introduction of the *TRAIL* gene into cancer cells can elicit apoptosis and suppress tumor growth *in vitro* and *in vivo* (6, 7). We also have demonstrated that nontransduced neighboring cancer cells can be killed by the *TRAIL* or *GFP/TRAIL* fusion genes through bystander effects (7). In a separate study, we recently demonstrated that the hTERT promoter, whose gene is highly active in >85% of human cancers but is inactive in most somatic cells (8), can be used to target proapoptotic genes to cancers (9). In this study, we show that the GAL4 gene regulatory system can be used to augment transgene expression from the hTERT promoter without losing the target specificity. As a result, we have created an adenoviral vector designated Ad/gTRAIL that expresses the *GFP/TRAIL* fusion gene driven by the hTERT promoter via GAL4 gene-regulatory components. Our study shows that treatment with Ad/gTRAIL elicited apoptosis in malignant cells and suppressed tumor growth *in vivo* with minimal toxicity to NHPHs.

Materials and Methods

Adenoviruses. Adenoviral vectors Ad/hTERT-LacZ, Ad/hTERT-GV16, Ad/CMV-LacZ, Ad/PGK-GV16, Ad/CMV-GFP, and Ad/GT-TRAIL were described previously (7, 9, 10). Ad/gTRAIL was constructed as described previously (10, 11). This vector contains two expression cassettes, one for the GFP/TRAIL fusion protein (7), whose gene is driven by a synthetic, minimal promoter composed of five sets of GAL4 binding sites and a TATAA sequence (GT promoter), and the other for GAL4/VP16, a transactivator, whose gene is driven by the hTERT promoter. The expansion, purification, titration, and quality analysis of all vectors used were performed as described previously (9) at the vector core facility of our institution. The titer and yield of Ad/gTRAIL was in the range of other E1-deleted adenoviral vectors.

Cell Lines and Human Hepatocytes. Human lung cancer cell lines A549 and H460 and human colon cancer cell lines DLD-1 and Lovo, were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, and glutamine. NHFBs (Clonetics, San Diego, CA) were cultured in media recommended by the manufacturer. NHPHs were either obtained from Applied Cell Biology Research Institute (Kirkland, WA) or isolated from normal, noncirrhotic liver tissues collected from surgical specimens from patients undergoing hepatic resection under a protocol approved by the institutional review board at The University of Texas M. D. Anderson Cancer Center. Collagenase digestion of liver specimens and culturing of primary human hepatocytes were performed as described previously (12).

***In Vitro* Gene Transfer.** The optimal MOI was determined by infecting each cell line with Ad/CMV-LacZ and assessing the expression of β-galactosidase via 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. MOIs that resulted in 50–80% of cells being stained blue were used in this experiment. These MOIs were 1000 particles for DLD-1, Lovo, A549, NHFBs, and primary human hepatocytes and 2000 particles for H460 cells. Unless otherwise specified, Ad/GT-TRAIL + Ad/PGK-GV16 was used as a positive

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[3] The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; ALT, alanine aminotransferase; AST, aspartate aminotransferase; CMV, cytomegalovirus; GFP, green fluorescent protein; GV16, GAL4/VP16 fusion protein; hTERT, human telomerase reverse transcriptase; GT, Gal4/TATA; MOI, multiplicity of infection; NHFB, normal human fibroblast; NHPH, normal human primary hepatocyte; PGK, 3-phosphoglycerate kinase; XTT, 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide; FACS, fluorescence-activated cell sorting; BGH, bovine growth hormone.

control, and Ad/CMV-GFP was used as a vector control. Cells treated with only PBS were used as a mock control.

Biochemical and Flow Cytometric Assays. Cell viability was determined by XTT assay using Cell Proliferation Kit II (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol as described previously (7, 9). Each experiment was performed in quadruplet and repeated at least twice. Fluorescence-activated cell sorting (FACS), Western blot analysis, and *in situ* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining were performed as described previously (7, 9).

Animal Experiments. Animal experiments were carried out in accordance with the Guidelines 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. Human colon carcinoma xenografts were established in nude mice (6–8 weeks old; Charles River Laboratories Inc., Wilmington, MA) by s.c. inoculation of 2×10^6 DLD-1 cells into the dorsal flank of each mouse. Intratumor injection of adenoviral vectors or PBS was performed when the tumors had reached 0.5 cm in diameter. Three intratumoral injections were given every 5 days at a dose of 6×10^{10} particles/injection/tumor in 200 μl of PBS. Ten mice from each group were followed up three times/week to measure tumor sizes by calipers. Tumor volume was calculated as follows: volume $= a \times b^2/2$ (*a,* largest diameter; *b*, smallest diameter; Refs. 7 and 9). Mice were killed according to institutional guidelines when the tumor reached 1.5 cm in diameter.

Toxicity after systemic gene delivery also was studied in 6–8-week-old BALB/c mice (Charles River Laboratories Inc.). In brief, mice were given i.v. injections of 6×10^{10} particles of adenovirus vectors in a total volume of 200 μl. At 2, 14, and 30 days after injection, three mice were killed by CO_2 inhalation. Various organs were then harvested for histopathological examination as described previously (7, 9). For serum liver function test, serum samples were collected from mice 2, 10, and 30 days after the treatment. Damage to hepatocytes was monitored by examining serum ALT and AST levels as reported previously (9).

Statistical Analysis. Differences among the treatment groups were assessed by ANOVA using Statistica software (StatSoft Inc., Tulsa, OK). Results of the experiments on tumor growth *in vivo* were analyzed by ANOVA, with a repeated measurement module. Survivals were analyzed by log-rank test using Statistica software. $P \leq 0.05$ was considered significant.

Results

Construction and Characterization of Ad/gTRAIL. We recently observed that the hTERT promoter can be used to impose the therapeutic effects of a proapoptotic gene on cancers (9). We also have observed that transgene expression from the carcinoembryonic antigen promoter can be increased more than 20- to 100-fold *in vitro* and *in vivo* via a GAL4 gene-regulatory system without loss of the promoter's specificity (13). Our recent study also showed that the GAL4 gene-regulatory components can be used to enhance transgene expression from the hTERT promoter without compromising its specificity (data not shown). On the basis of these observations, we constructed a bicistronic adenoviral vector, Ad/gTRAIL. This bicistronic vector expresses the *GFP/TRAIL* fusion gene from the hTERT promoter via the GAL4 gene-regulatory system (Fig. 1*A*). Although this vector initially was constructed in 293 cells developed in our laboratory that express the trans-repressor GAL4/KRAB-A (Ref. 14; gift of Dr. J. V. Bonventre, Harvard University, Boston, MA), the vector can be expanded and purified from regular 293 cells without any problem.

We characterized this vector's functionality in the human colon cancer cell line DLD-1, which we previously had found to be very sensitive to the *TRAIL* gene (7). We infected DLD-1 cells with a control vector expressing GFP from the CMV early promoter, Ad/CMV-GFP, at a MOI of 1000 viral particles/cell. This resulted in about 50% GFP-positive cells, as detected under a fluorescence microscope, but cell death did not occur. However, infecting these cells with the same dose of Ad/gTRAIL resulted in the same level of GFP-positive cells, yet >90% of the Ad/gTRAIL-treated cells were killed 48 h after treatment, as judged by morphology changes (Fig. 1*B*). Thus, treatment with Ad/gTRAIL can effectively kill TRAIL-sensitive cancer cells.

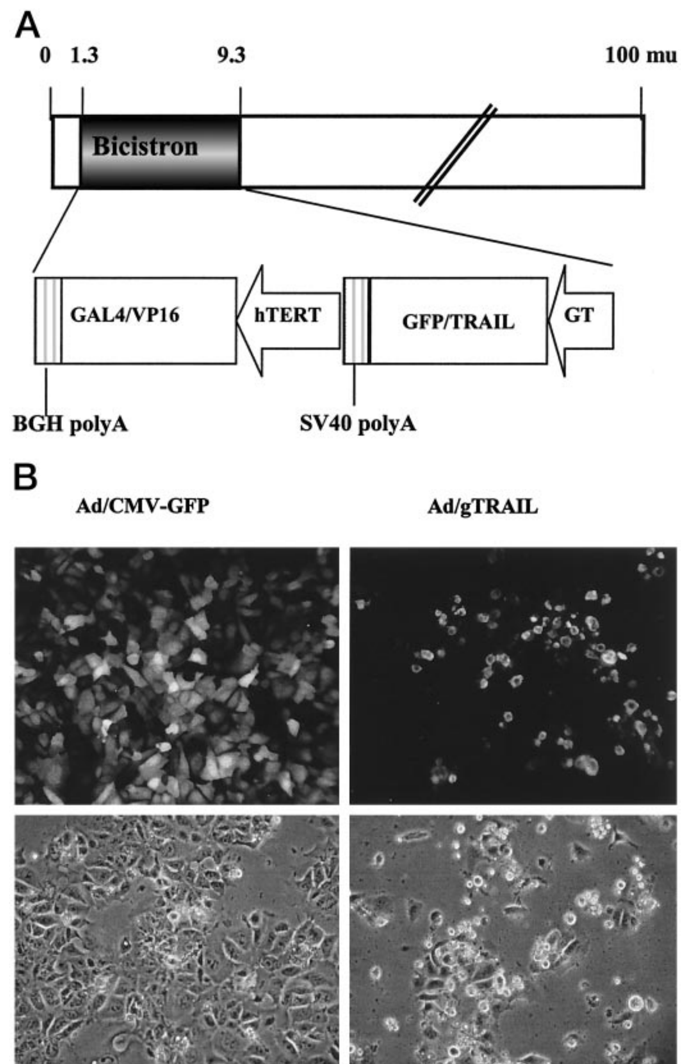

Fig. 1. *A,* diagram of Ad/gTRAIL. The E1 region (map unit 1.3–9.3) of human adenovirus type 5 is replaced by therapeutic sequences composed of expression cassettes for the *GAL/VP16* and *GFP/TRAIL* genes. Polyadenylation signal sequences from BGH and SV40 genes are used for these cassettes. *B,* DLD-1 cells were treated with the same doses of Ad/CMV-GFP or Ad/gTRAIL. Expression of GFP or its fusion protein (*top panels*) was revealed under a fluorescent microscope and compared with cell density and morphology (*bottom panels*).

Transgene Expression and Toxicity of Ad/gTRAIL in Normal Human Primary Hepatocytes. We evaluated the effects of Ad/gTRAIL on NHFBs or NHPHs isolated from surgical specimens. For this purpose, NHPHs or NHFBs were treated with either PBS, Ad/CMV-GFP, Ad/gTRAIL, or Ad/GT-TRAIL + Ad/PGK-GV16 at a total MOI of 1000 viral particles/cell. When the binary system was used, the total dose remained the same, whereas the ratio for the two vectors was set to 1:1. Two days later, cells were harvested and divided into two parts. One part was used to analyze GFP expression, and the second part was used to quantify apoptotic cells by flow cytometric assay, as described above. Only treatment with Ad/CMV-GFP resulted in >50% GFP-positive cells in either NHPHs or NHFBs. In contrast, treatment with Ad/gTRAIL resulted in <1% GFP-positive cells, similar to what is seen in NHPH or NHFB cells treated with PBS or Ad/GT-TRAIL + Ad/PGK-GV16 (the latter does not have a GFP component; therefore, transgene expression cannot be

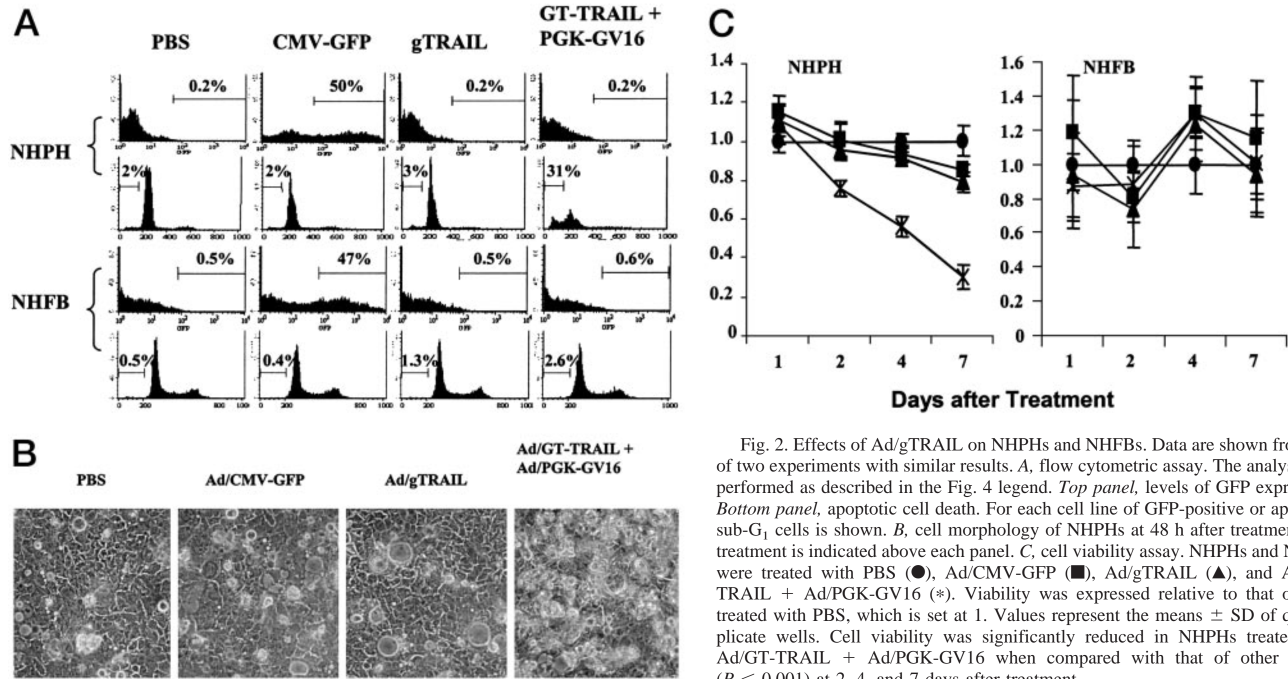

Fig. 2. Effects of Ad/gTRAIL on NHPHs and NHFBs. Data are shown from one of two experiments with similar results. *A,* flow cytometric assay. The analysis was performed as described in the Fig. 4 legend. *Top panel,* levels of GFP expression. *Bottom panel,* apoptotic cell death. For each cell line of GFP-positive or apoptotic sub-G_1 cells is shown. *B,* cell morphology of NHPHs at 48 h after treatment. The treatment is indicated above each panel. *C,* cell viability assay. NHPHs and NHFBs were treated with PBS (●), Ad/CMV-GFP (■), Ad/gTRAIL (▲), and Ad/GT-TRAIL + Ad/PGK-GV16 (*). Viability was expressed relative to that of cells treated with PBS, which is set at 1. Values represent the means ± SD of quadruplicate wells. Cell viability was significantly reduced in NHPHs treated with Ad/GT-TRAIL + Ad/PGK-GV16 when compared with that of other groups ($P < 0.001$) at 2, 4, and 7 days after treatment.

detected by GFP assay; Fig. 2*A*). Cytometric analysis of apoptosis showed that treatment with Ad/gTRAIL or Ad/GT-TRAIL + Ad/PGK-GV16 resulted in only background levels of apoptosis in fibroblasts. This finding is consistent with our previous observation that treatment with the *TRAIL*-expressing vectors did not result in cell death in NHFBs. Interestingly, however, treatment with Ad/GT-TRAIL + Ad/PGK-GV16 led to a dramatic increase in apoptotic cells (>30%) in NHPHs. In comparison, treatment with Ad/gTRAIL resulted in only a background level of cell death, similar to that seen in cells treated with PBS or control vector (Fig. 2*A*). These data indicate that NHPHs are susceptible to full-length human *TRAIL* molecules and that the hTERT promoter can be used to prevent expression of therapeutic genes in normal human hepatocytes, thereby preventing possible normal tissue toxicity. This observation was further supported by the fact that treatment of NHPHs with Ad/GT-TRAIL + Ad/PGK-GV16, but not with Ad/gTRAIL, Ad/CMV-GFP, or Ad/GT-LacZ + Ad/PGK-GV16, resulted in typical apoptotic morphological changes as revealed by microscopic study or cell viability loss as revealed by XTT assay (Fig. 2, *B* and *C*). Because treatment with Ad/GT-LacZ + Ad/PGK-GV16 at the same dose and vector ratio has no toxic effect on NHPHs, the toxicity observed in NHPHs after treatment with Ad/GT-TRAIL + Ad/PGK-GV16 should be TRAIL related rather than GV16 related. The same results were observed when using primary hepatocytes obtained from Applied Cell Biology Research Institute (data not shown).

Transgene Expression and Toxicity of Ad/gTRAIL after Systemic Administration. We also investigated levels of transgene expression in the liver and the possible toxicity of Ad/gTRAIL after systemic administration. For this purpose, adult BALB/c mice (6–8 weeks old) were infused with PBS, Ad/CMV-GFP, Ad/gTRAIL, and Ad/GT-TRAIL + Ad/PGK-GV16 via the tail vein (ratio 1:1 in this group) at a total dose of 6×10^{10} particles/mouse. Our previous study had shown that >90% of liver cells are transduced at this dose (9). Animals were sacrificed at 2, 14, and 30 days after injection. Liver, spleen, lung, heart, pancreas, kidney, intestine, gonad, and brain were harvested for histopathological examination. No significant microscopic lesions were observed in any animals at 2 days after treatment. By 2 weeks, all animals treated with adenoviral vectors showed lymphoid hyperplasia in the spleen and inflammatory cell (lymphocytes, plasma cells, and neutrophils) infiltration in some portal areas in the liver. In addition, animals treated with Ad/GT-TRAIL + Ad/PGK-GV16 showed scattered necrotic hepatocytes and had numerous binucleated or trinucleated hepatocytes (polyloidy) and hepatocytes with large irregular-shaped nuclei (karyomegaly). These abnormal changes in hepatocytes had resolved by day 30. The results of serum liver enzyme assays were consistent with histopathological changes observed in the liver. AST and ALT levels were within normal ranges at day 2 and day 30 but were elevated at day 14 in animals treated with adenoviral vectors (Fig. 3*A*). The elevation was more pronounced in animals treated with Ad/GT-TRAIL + Ad/PGK-GV16. Of note, E1-deleted adenoviral vectors are immunogenic and will cause a subacute inflammatory response in the livers of immunocompetent animals after systemic delivery (15, 16). Because a similar degree of inflammatory response was observed in animals treated with Ad/CMV-GFP or Ad/gTRAIL, this response is regarded as vector related rather than as transgene related. Interestingly, the inflammatory response was more severe in animals treated with Ad/GT-TRAIL + Ad/PGK-GV16. The significance of this finding is not yet clear, however, this phenomenon was not observed in nude mice (7).

Because systemic administration of adenoviral vector resulted in transduction of liver cells (9, 17), liver samples from the above-mentioned animals also were collected for Western blot analysis of GFP or GFP/TRAIL fusion protein expression and for PCR analysis of the viral genome. For animals treated with Ad/CMV-GFP, Western blot analysis with anti-GFP polyclonal antibody showed a strong GFP band by day 2 (Fig. 3*B*), which became much weaker by day 14 (data not shown). The GFP was not detectable by Western blot analysis by day 30. This result is consistent with observations that transgene expression from adenoviral vectors is transient in immunocompetent animals (17). In animals treated with Ad/gTRAIL, however, no transgene expression was detected by Western blot analysis in any animals at any of the time points tested. Of note, the GFP antibody used can readily detect the GFP/TRAIL fusion protein in DLD-1 cells treated with Ad/gTRAIL. Furthermore, the same level of viral DNA was

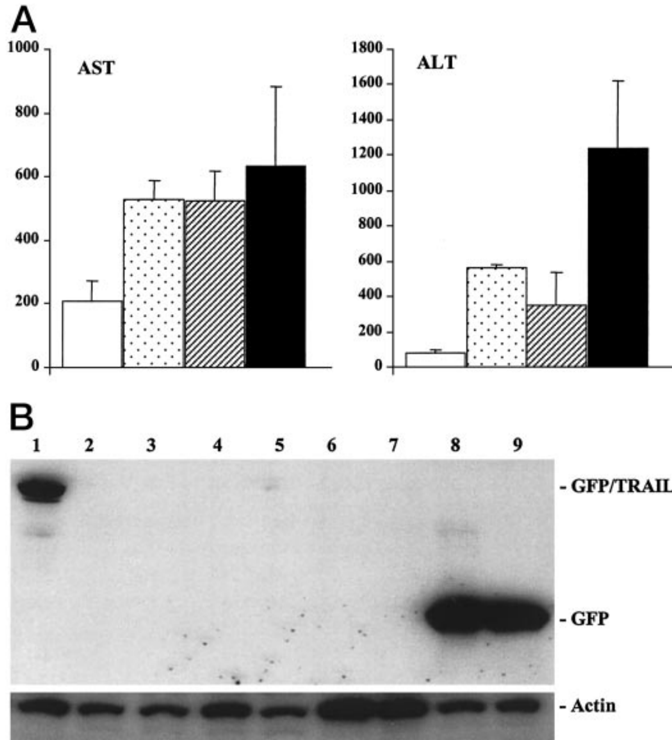

Fig. 3. *In vivo* assessment after systemic delivery in BALB/c mice. *A,* activity of serum liver enzymes AST and ALT at day 14. □, PBS; ▨, Ad/CMV-GFP; ▨, Ad/gTRAIL; ■, Ad/GT-TRAIL + Ad/PGK-GV16. *B,* Western blot analysis of liver samples collected at 2 days after treatment. *Lane 1,* DLD-1 cancer cells treated with Ad/gTRAIL (positive control for GFP/TRAIL); *Lanes 2–4,* DLD-1 cancer cells treated with Ad/GT-TRAIL + Ad/PGK-GV16; *Lanes 5–7,* DLD-1 cancer cells treated with Ad/gTRAIL; *Lanes 8* and *9,* DLD-1 cancer cells treated with Ad/CMV-GFP.

detected in the livers of animals treated with either Ad/CMV-GFP or Ad/gTRAIL by a semiquantitative PCR analysis, suggesting that the lack of transgene expression by Ad/gTRAIL was not caused by an artifact. These findings suggest that the hTERT promoter can be used to prevent expression of the *GFP/TRAIL* gene in the liver after systemic administration of Ad/gTRAIL. This is consistent with our previous observation that the hTERT promoter can be used to prevent *Bax* gene-related liver toxicity after systemic administration of the *Bax*-expressing binary adenoviral vectors (9).

Transgene Expression and Apoptosis Induction by Ad/gTRAIL in Cancer Cells *in Vitro*. To evaluate the levels of transgene expression and apoptosis induction of Ad/gTRAIL in cancer cells, we treated human lung cancer cell lines (A549 and H460) and human colon cancer cell lines (DLD-1 and Lovo) with Ad/gTRAIL, Ad/CMV-GFP, or Ad/GT-TRAIL + Ad/PGK-GV16 at a fixed total MOI as described in "Materials and Methods." Two days later, cells were harvested and analyzed for GFP expression and apoptosis by FACS. Treatment with Ad/CMV-GFP or Ad/gTRAIL resulted in similar levels of GFP-positive cells (70–90%) in all of the cell lines tested, suggesting that levels of transgene expression for the two vectors were similar in these cancer cell lines (Fig. 4*A*). However, treatment with Ad/gTRAIL dramatically increased the number of apoptotic cells (Fig. 4*A*), a result that is comparable with findings for cells treated with a binary vector system expressing wild-type human *TRAIL* (Ad/GT-TRAIL + Ad/PGK-GV16; Ref. 7). In comparison, treatment with Ad/CMV-GFP resulted in only background levels of apoptosis, and treatment with Ad/GT-TRAIL + Ad/PGK-GV16 resulted in only background levels of GFP-positive cells because the binary vectors did not contain any GFP component. These results demonstrate that treatment with Ad/gTRAIL can elicit high levels of transgene expression and high levels of apoptosis in cancer cells. The cell killing effects of Ad/gTRAIL in cancer cells were further documented by XTT assay (Fig. 4*B*).

Suppression of Tumor Growth by Ad/gTRAIL *in Vivo*. To test whether intralesional administration of Ad/gTRAIL can suppress tumor growth, we compared the antitumor effect of Ad/gTRAIL with that of the binary vectors (Fig. 5*A*). A direct comparison showed that

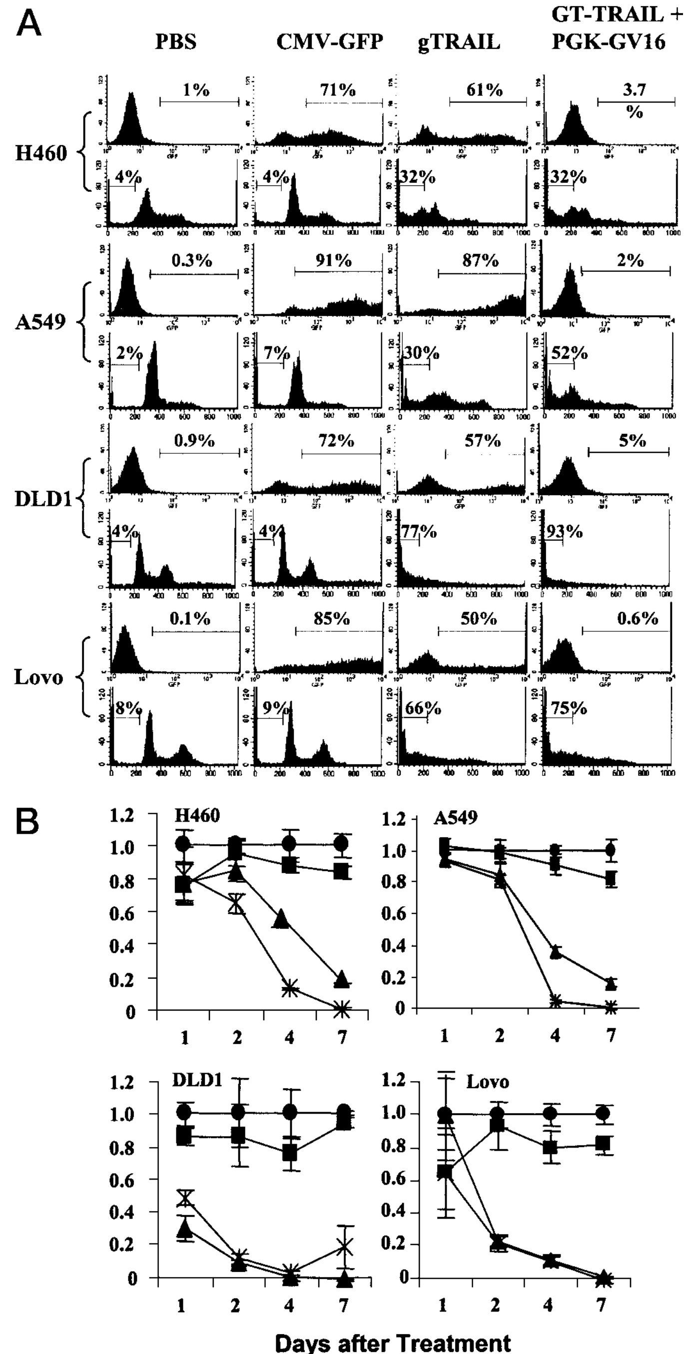

Fig. 4. Transgene expression and cell killing effects of Ad/gTRAIL *in vitro*. Data are shown from one of two experiments with similar results. *A,* flow cytometric assay. H460, A549, DLD-1, and Lovo cells were harvested 48 h after treatment, as indicated above each column. Levels of GFP expression (*top panel*) and apoptotic cell death (*bottom panel*) for each cell line were determined by FACS. Percentage of GFP-positive or apoptotic sub-G_1 cells is shown. *B,* cell viability as determined by XTT assay. Cells were treated with PBS (●), Ad/CMV-GFP (■), Ad/gTRAIL (▲), and Ad/GT-TRAIL + Ad/PGK-GV16 (*). Viability is expressed relative to that of cells treated with PBS, which was set at 1. Values represent the means ± SD of quadruplicate wells. In all four cell lines, viability after treatment with TRAIL-expressing vectors *versus* control vectors differed significantly ($P < 0.001$) at 4 and 7 days after treatment.

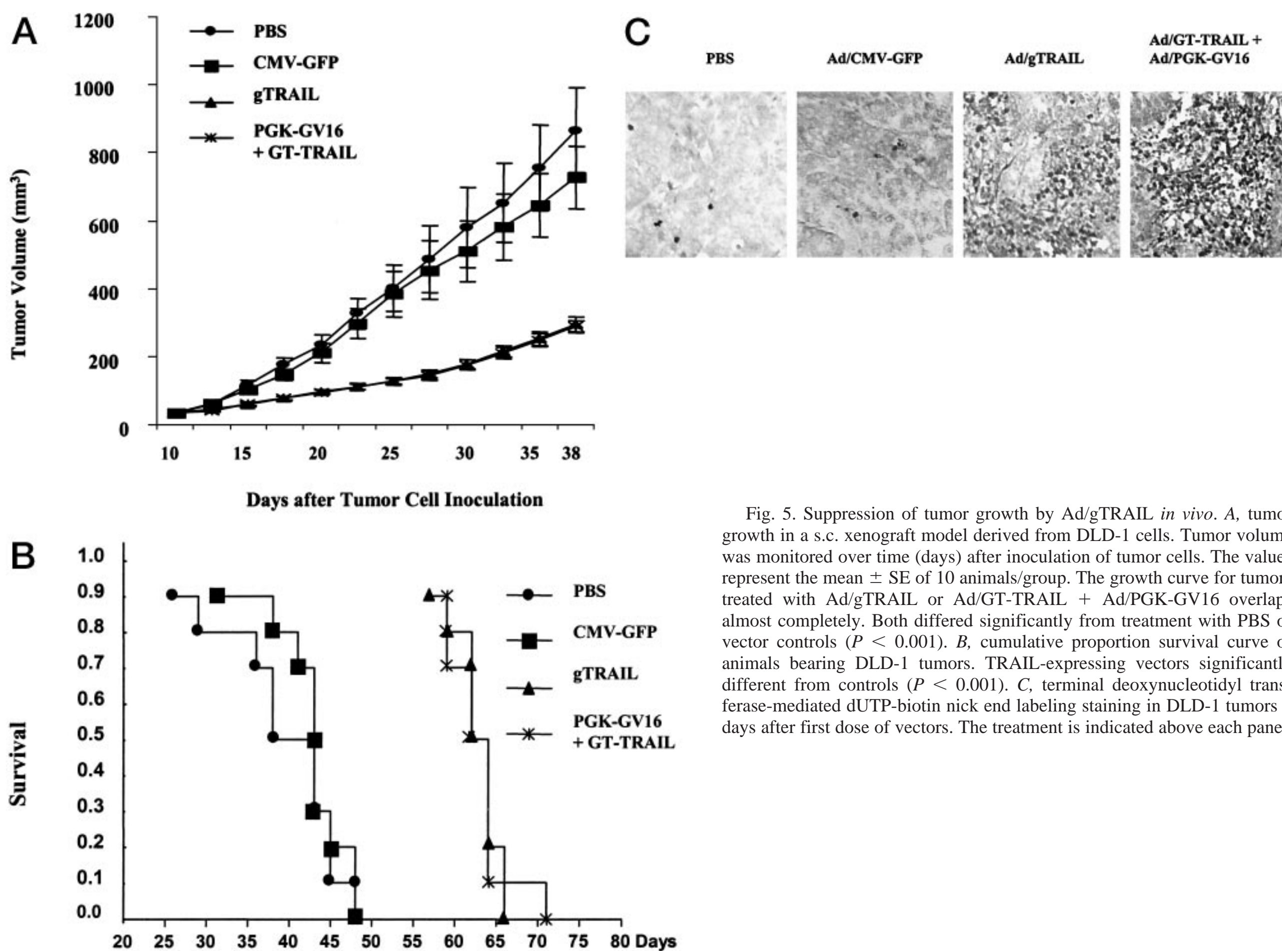

Fig. 5. Suppression of tumor growth by Ad/gTRAIL *in vivo*. *A,* tumor growth in a s.c. xenograft model derived from DLD-1 cells. Tumor volume was monitored over time (days) after inoculation of tumor cells. The values represent the mean ± SE of 10 animals/group. The growth curve for tumors treated with Ad/gTRAIL or Ad/GT-TRAIL + Ad/PGK-GV16 overlaps almost completely. Both differed significantly from treatment with PBS or vector controls ($P < 0.001$). *B,* cumulative proportion survival curve of animals bearing DLD-1 tumors. TRAIL-expressing vectors significantly different from controls ($P < 0.001$). *C,* terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling staining in DLD-1 tumors 2 days after first dose of vectors. The treatment is indicated above each panel.

intralesional administration of Ad/gTRAIL resulted in the same antitumor effects as those of Ad/GT-TRAIL + Ad/PGK-GV16, suggesting that Ad/gTRAIL is as effective as Ad/GT-TRAIL + Ad/PGK-GV16 in terms of antitumor activity *in vivo*. In comparison, tumors treated with Ad/CMV-GFP grew as fast as those treated with PBS. The treatment with TRAIL-expressing vectors also led to a survival advantage (Fig. 5*B*). The mean survival durations after treatment with PBS, Ad/CMV-GFP, Ad/gTRAIL and Ad/GT-TRAIL + Ad/PGK-GV16 were 39, 42, 62.5, and 63 days, respectively. Posttreatment histochemical examination of tumor tissues supported these results. Treatment with Ad/gTRAIL or Ad/GT-TRAIL + Ad/PGK-GV16 dramatically increased apoptosis, whereas treatment with Ad/CMV-GFP or PBS resulted in only background apoptosis (Fig. 5*C*).

Discussion

Recombinant TRAIL proteins have been intensively investigated for cancer treatment *in vitro* and in animal models (2). However, reports concerning the possible toxic effects of soluble TRAIL proteins on normal human hepatocytes, brain tissue, and certain epithelial cells have spurred more study of recombinant TRAIL protein's toxicity (3). A recent report found evidence that the observed toxicity could be caused by tagged histidine or leucine in recombinant TRAIL proteins (5). In this study, we found that direct transfer of the wild-type, full-length coding sequence of the human *TRAIL* gene to cultured primary human hepatocytes triggered massive apoptosis of these cells. Although the mechanism and significance of this observation remain unclear, this result indicates that membrane-bound TRAIL may behave differently from soluble TRAIL. Because cultured primary hepatocytes are dramatically different from hepatocytes *in situ* (18), the importance of the hepatocyte toxicity stemming from full-length, membrane-bound TRAIL that we observed is not yet clear. However, because liver is a complex organ composed of multiple cell types (18) and because the *TRAIL* gene is normally not expressed in human liver, the issue of liver toxicity resulting from the *TRAIL* gene overexpression or from the recombinant TRAIL proteins should not be overlooked.

We recently have shown that the hTERT promoter is highly active in most cancer cells tested, but not in normal cells or in normal mouse tissues (9). However, in most cancer cells, the hTERT promoter activity is also >10-fold lower than that of CMV promoter (9). More recently, we found that transgene expression from a tumor-specific promoter can be augmented by using GAL4 gene-regulatory system (13). The same approach can be used to enhance the transgene expression from the hTERT promoter. This may be explained by a potent transactivating ability of GAL4/VP16 (10). A minimal amount of GAL4/VP16 is sufficient to activate its target, the GAL4/TATA promoter, leading to enhanced expression of the therapeutic gene in the target cells. We found that this approach is necessary to achieve high therapeutic levels of transgene expression (data not shown). The fact that treatment of NHPHs with Ad/gTRAIL resulted in no detectable transgene expression and in minimal toxicity suggests that the hTERT promoter in combination with GAL4 gene-regulatory components can effectively prevent the toxic effects of a therapeutic gene in some normal tissues. Furthermore, *in vivo* study on mice showed no transgene expression from Ad/gTRAIL in liver after system administration of a high dose of Ad/gTRAIL. Because normal human liver

tissue expresses no hTERT or minimal hTERT (19, 20), it is expected that the hTERT promoter is either silent or minimally active in normal human liver tissue. The *in vivo* antitumor activity and survival advantages from Ad/gTRAIL suggest that Ad/gTRAIL is a potent antitumor agent with a broad spectrum. However, these results are not completely unexpected because TRAIL is effective in a variety of tumors, and the hTERT promoter is active in 85% of human cancers. Together, our results suggest that with the combined advantages of the *TRAIL* gene and the hTERT target, Ad/gTRAIL can be a potent agent for the treatment of cancers.

Acknowledgments

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