

Antitumor Activity and Bystander Effects of the Tumor Necrosis Factor-related Apoptosis-inducing Ligand (*TRAIL*) Gene¹

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ABSTRACT

Tumor necrosis factor-related apoptosis-inducing ligand (*TRAIL*) has been reported to specifically kill malignant cells but to be relatively nontoxic to normal cells. To evaluate the antitumor activity and therapeutic value of the *TRAIL* gene, we constructed adenoviral vectors expressing the human *TRAIL* gene and transferred them into malignant cells *in vitro* and tumors *in vivo*. The *in vitro* transfer elicited apoptosis, as demonstrated by the quantification of viable or apoptotic cells and by the analysis of activation of pro-caspase-8 and cleavage of poly(ADP-ribose) polymerase. The intratumoral delivery elicited tumor cell apoptosis and suppressed tumor growth. In comparison with *Bax* gene treatment, which is toxic to normal cells, *TRAIL* gene treatment caused no detectable toxicity in cultured normal fibroblasts nor in mouse hepatocytes after systemic gene delivery. Furthermore, coculture of cancer cells expressing *TRAIL* with those expressing green fluorescent protein (GFP) resulted in apoptosis of both cells, whereas coculture of *Bax*-expressing cells with GFP-expressing cells resulted in the cell death of the *Bax*-expressing cells only, which suggested that the transfer of the *TRAIL* gene resulted in bystander effects. Moreover, culture of cells with medium from *TRAIL*-expressing cells showed the proapoptotic activity and bystander effect of the *TRAIL* gene to be not transferable with medium. To further demonstrate the bystander effect of the *TRAIL* gene, we constructed plasmid vectors encoding GFP-*TRAIL* or GFP-*Bik* chimeric proteins. Transfection of the GFP-*TRAIL* gene into cancer cells resulted in the death of GFP-positive cells and their neighbors, whereas transfection of the GFP-*Bik* gene killed GFP-positive cells only. Finally, GFP-*TRAIL* genes, transfected into normal human fibroblasts or bronchial epithelial cells, did not kill such cells, whereas transfected GFP-*Bik* genes did. Thus, the direct transfer of the *TRAIL* gene led to selective killing of malignant cells with bystander effect, which suggests that the *TRAIL* gene could be valuable for treatment for cancers. Together, these results suggest that delivering the *TRAIL* gene to cancerous cells may be an alternative approach to cancer treatment.

INTRODUCTION

In the search for tumor-specific agents of gene therapy, one promising agent has arisen from among the TNF⁴ family of factors. This

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⁴ The abbreviations used are: TNF, tumor necrosis factor; *TRAIL*, TNF-related apoptosis-inducing ligand; GT, GAL4/TATA; GV16, GAL4/VP16 fusion protein; PGK, 3-phosphoglycerate kinase; PARP, poly(ADP-ribose) polymerase; XTT, 2,3-bis-(2-

agent, *TRAIL*/Apo-2L, was first identified through a search of an expressed sequence tag (EST) database using a conserved sequence contained in many TNF family members (1, 2). *TRAIL* is a type II transmembrane protein whose extracellular region forms a soluble molecule on cleavage (1, 2). Both membrane-bound *TRAIL* and soluble *TRAIL* rapidly induce apoptosis in a wide variety of transformed cell lines via interaction with the death receptors DR4/*TRAIL*-R1 and DR5/*TRAIL*-R2 (3, 4). However, unlike its relatives, TNF and FasL, whose expression is tightly regulated and which are only transiently expressed on some activated cells, *TRAIL* is expressed constitutively in many normal tissues including lymphocytes, spleen, thymus, prostate, ovary, and intestine (although not in brain, liver, or testis; 1). In fact, the presence of *TRAIL* in normal tissues suggests that normal cells contain mechanisms that protect them from apoptosis induction by *TRAIL*. Recent studies have demonstrated the existence of antagonistic decoy receptors, DcR1, DcR2, and osteoprotegerin that may compete with DR4 and DR5 for *TRAIL* binding, thereby putatively protecting normal cells from the cytotoxic effects of *TRAIL* (reviewed in (5, 6)). Thus, *TRAIL* appears to induce apoptotic cell death only in tumorigenic or transformed cells and not in normal cells (7, 8). Recent studies showed that repeated *in vivo* injection of recombinant and biologically active *TRAIL* induced tumor cell apoptosis, suppressed tumor progression, and improved survival in mice bearing solid tumors, but caused no detectable toxicity in them (7, 8). Furthermore, *TRAIL* cooperated synergistically with chemotherapeutic drugs to cause substantial tumor regression and, in some cases, complete tumor ablation (7, 9, 10). Together, these data suggest that *TRAIL* may act as a potent anticancer agent while causing no significant toxicity to normal tissues.

The promising results obtained using recombinant *TRAIL* protein suggested therapeutic value of the *TRAIL* gene. Directly introducing the *TRAIL* gene into tumor cells would be used to elicit antitumor effects. Furthermore, local intratumoral expression of the *TRAIL* gene would minimize the potential toxicity of systemically administered *TRAIL*. To evaluate the therapeutic value of the approaches using the *TRAIL* gene, we constructed adenoviral vectors expressing the human *TRAIL* gene using a binary vector system that allows expression of a highly apoptotic gene (11). We then assessed the antitumor activities of the *TRAIL in vitro* in cultured cells and *in vivo* in an experimental tumor model. Finally, we explored the bystander effect of the *TRAIL* gene on human cancer cells and normal cells using adenovirus- or plasmid-mediated gene transfer. Our results demonstrated that the direct transfer of the *TRAIL* gene into cancer cells elicited cell death, bystander effects, and suppressed tumor growth *in vitro* and *in vivo* while exerting few toxic effects on normal cells

methoxy-4-nitro-5-sulphenyl)-(2H)-tetrazolium-5-carboxanilide; Tdt, terminal deoxynucleotidyl transferase; TUNEL, Tdt-mediated dUTP-biotin nick end labeling; AST, aspartate transaminase; ALT, alanine transaminase; GFP, green fluorescent protein; NHLF, normal human lung fibroblast; NHBE, normal human bronchial epithelial cell; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; MOI, multiplicity/multiplicities of infection; X-gal, 5-bromo-4-chloro-3-indolyl- β -D-galactoside; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PI, propidium iodide.

tested. The antitumor activity and the bystander effect of the *TRAIL* gene were not transferable via medium, suggest that the antitumor activity and bystander effect are mainly elicited through membrane-bound *TRAIL*.

MATERIALS AND METHODS

Cell Lines. Human lung cancer cell lines A549 and H460 maintained in our laboratory were grown in Ham's/F12 and RPMI 1640 media, respectively, supplemented with 10% FBS and antibiotics. Human colon cancer cell line DLD-1 obtained from Dr. T. Fujiwara (Okayama University, Okayama, Japan) were grown in RPMI 1640 supplemented with 10% FBS and antibiotics. NHLF cells, obtained from Clonetics (San Diego, CA) were grown in culture medium supplied by the manufacturer.

Adenoviruses. Adenoviral vectors Ad/PGK-GV16, Ad/GT-LacZ, Ad/GT-Bax, and AdE1 were constructed as described previously (12, 13). Ad/GT-*TRAIL*, an adenoviral vector expressing *TRAIL*, was also constructed as described previously (12). Briefly, a cDNA containing the entire coding sequence of human *TRAIL* (Invivogen, San Diego, CA) was inserted into an expression cassette driven by a GT promoter to generate shuttle plasmid pAD/GT-*TRAIL*. This shuttle plasmid was then cotransfected into 293 cells along with a 35-kb *Clal* fragment from adenovirus type 5. Then, recombinant vector Ad/GT-*TRAIL* was generated by homologous recombination and plaque-purified. The sequence of its expression cassette was then confirmed by automatic DNA sequencing in the DNA sequencing core facility at the University of Texas M. D. Anderson Cancer Center. The expansion, purification, titration, and quality analysis of all of the vectors used were performed at the vector core facility of our institution as described previously (12, 13). All of the viral preparations were found to be free of the E1+ adenovirus by PCR assay (14) and endotoxin by testing with a *Limulus* amoebocyte lysate endotoxin detection kit (BioWhittaker, Walkersville, MD). The titer determined by the absorbency of the dissociated virus at $A_{260\text{ nm}}$ (one $A_{260\text{ nm}}$ unit = 10^{12} viral particles/ml) was used in this study, whereas titers determined by plaque assay were used as additive information. Particles:plaque ratios were usually between 30:1 and 100:1.

Plasmid Constructs. A plasmid vector expressing a fusion protein consisting of GFP and full length of human *TRAIL* (pEGFP-*TRAIL*) was constructed by placing *TRAIL* cDNA into the 3'-end of GFP in a pEGFP/C3 expression vector (Clontech Laboratories, Inc., Palo Alto, CA). To compare the effect of the *TRAIL* gene, we also constructed a plasmid vector expressing a fusion protein consisting of GFP and human Bik (Ref. 15; pEGFP-Bik) in the same manner. Each expression cassette consisted of the human cytomegalovirus promoter and the polyadenylation signal sequence from SV40. (The detailed cloning procedure is available on request.) Automatic DNA sequencing was used to analyze DNA sequences at their junctions with GFP and to confirm the authenticity of each construct's sequence.

In Vitro Gene Transfer. As determined in preliminary experiments, cells were coinfecting with Ad/GT-*TRAIL* or Ad/GT-LacZ and Ad/PGK-GV16 at a ratio of 1:1. The optimal MOI was determined by infecting each cell line with Ad/GT-LacZ + Ad/PGK-GV16 and assessing the expression of β -galactosidase via X-gal staining. Those MOI that resulted in >80% of cells being stained blue were used in this experiment. These MOI were 1000 particles for DLD-1, A549, and NHLF cells and 2000 particles for H460 cells. Unless otherwise specified, Ad/GT-LacZ + Ad/PGK-GV16 was used as the vector control for Ad/GT-*TRAIL* + Ad/PGK-GV16. Cells treated with PBS only were used as a mock control. Plasmid transfection was performed using Fugene-6 (Roche Molecular Biochemicals, Indianapolis, IN).

RNase Protection Assays. For each experiment, 2,000,000 cells were plated on 100-mm dishes and infected with adenovirus. Twenty-four h after infection, cells were harvested and washed with PBS. The RNA was extracted from the cells using Trizol Reagent (Life Technology Inc., Gaithersburg, MD) following the manufacturer's instructions. RNase protection assay was performed using 15 μ g of total RNA and the RiboQuant Multiprobe RNase Protection Assay system (PharMingen, San Diego, CA) according to the manufacturer's protocol. 32 P-labeled antisense RNA probes were prepared using a set of human apoptosis hAPO-3C templates from PharMingen. The transcripts were visualized by autoradiography with a Storm PhosphorImager (Molecular Dynamics, Sunnyvale, CA). For each sample, RNA loading was normalized to the signal of GAPDH.

Biochemical Analysis. Cell viability was assessed using the Cell Proliferation Kit II (XTT; Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's protocol. X-gal staining of cultured cells and sectioned tissues was performed as described previously (13). Western blot analysis was performed as described previously (11), using the following primary antibodies: anti-PARP (4C10-5; PharMingen); anti-pro-caspase-8 (B9-2; PharMingen); anti-caspase-8 p20 (c-20; Santa Cruz, Santa Cruz, CA); and anti-actin (AC-15; Sigma, St. Louis, MO). ELISA was performed as described previously (16). Briefly, recombinant human *TRAIL* (Alexis Corporation, San Diego, CA) or media from cell cultures were diluted with 0.1 M NaHCO_3 (pH 9.5), and coated onto a 96-well plate. After incubation with blocking buffer [50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.05% Tween 20, and 5% Carnation low-fat milk] at 37°C for 1 h, the plate was incubated with blocking buffer containing a mouse monoclonal anti-*TRAIL* antibody (B35-1; PharMingen) for 1 h at 37°C. Bound antibody was detected by incubation with goat antimouse IgG and IgM (H+L) antibodies conjugated to horseradish peroxidase (Amersham, Piscataway, NJ). Enzymatic activity was determined using 3,3',5,5'-tetramethyl-benzidine dihydrochloride (TMB) tablets (Sigma). Twenty-five ng/ml of recombinant *TRAIL* in medium was readily detected with this ELISA.

FACS. FACS analysis for cell surface molecules was performed as described previously (17). In brief, cells were harvested by trypsinization at 24 h after treatment and were washed twice with PBS containing 1% FBS. Then 1×10^6 cells, suspended in 100 μ l PBS, were incubated at 4°C for 1 h with a rabbit polyclonal anti-*TRAIL* antibody (H257, Santa Cruz) at a concentration of 10 μ l/ml. After washing twice with 1% FBS/PBS, the cells were incubated in the dark at 4°C for 30 min with a FITC-labeled goat antirabbit immunoglobulin antibody (PharMingen) at a concentration of 10 μ l/ml. After washing twice with 1% FBS/PBS, the cells were suspended in 1% formaldehyde in PBS and subjected to FACS analysis. Normal rabbit IgG (Santa Cruz) was used as a control for primary antibodies; the levels detected by this control antibody were used as a basal background.

Bystander Effect Assay. The bystander effect of the *TRAIL* gene was assayed by flow cytometry as follows. Target cells were created by plating 3,000,000 cells on 60-mm dishes and infecting with Ad/CMV-GFP at 500 MOI. The next day, after GFP expression was confirmed in >90% cells under a fluorescence microscope, the cells were rinsed with PBS three times to remove residual free adenoviruses. Effector cells were created by plating 3,000,000 cells on 60-mm dishes and infecting with only Ad/GT-*TRAIL* at 500 MOI so that they could not express *TRAIL*. The next day, they were washed three times with PBS to remove residual free adenoviruses. Both target and effector cells were then trypsinized and washed once with growth medium. One million target and 1,000,000 effector cells were mixed and plated on each well of a 6-well plate. Ad/PGK-GV16 was then put into each well at 500 MOI to induce expression of the *TRAIL* gene. Forty-eight h later, PI was added to the medium at 5 μ g/ml to stain membrane-disrupted dead cells. Both floating and adherent cells were harvested and subjected to flow cytometry. GFP (+) cells were regarded as target cells, and GFP (-) cells were regarded as effector cells. PI-negative cells were regarded as living cells; PI-positive cells were regarded as dead ones.

Mouse Experiments. Mouse experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 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.) by s.c. inoculation of 1×10^6 DLD-1 cells into the dorsal flank of each mouse. Intratumoral injection of adenoviral vectors or PBS was usually initiated on day 10, when tumors had reached 0.5 cm in diameter. Three intratumoral injections were given once a week at a dose of 6×10^{10} particles/injection/tumor. Ten mice/group were followed twice a week by measuring the sizes of the s.c. tumors. In brief, calipers were used to determine the largest (*a*) and smallest (*b*) diameters, and tumor volume was then calculated as volume = $a \times b^2/2$ (13). When a mouse's tumor reached 1.5 cm in diameter, the mouse was killed according to institutional guidelines.

Toxicity after systemic gene delivery was also studied on nude mice (6–8 week-old; 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 μ l. At 4, 13, and 31 days after injection, three mice were killed by CO_2 inhalation. Various organs (brain, heart, lung, liver, intestine, spleen, pancreas,

ovary, kidney, and adrenal gland) were then harvested and fixed in 3.8% formalin in PBS. These tissues were then sectioned, stained with H&E, and examined histopathologically in the Department of Veterinary Histopathology at our institution. For analysis of liver function, serum samples were collected from living mice 2, 10, and 30 days after the treatment. Damage to hepatocytes was monitored by examining serum ALT and AST levels as reported previously (13).

Tumor Apoptosis Assay by TUNEL Staining. Apoptosis inside of tumors was also assessed by *in situ* TUNEL staining. For *in situ* TUNEL, analysis paraffin-embedded sections of tumor samples were deparaffinized and dehydrated. The slides were incubated in 3% H₂O₂ in methanol at room temperature for 10 min and then with 0.02% protease (P6911; Sigma)/PBS at 37°C for 30 min. The slides were then incubated with reaction buffer containing 0.17 units/ μ l Tdt, 0.2 nmol biotin-16-dUTP, and 2.5 mM cobalt chloride (all from Roche Molecular Biochemicals) at 37°C for 1 h. The reaction was terminated by transferring the slides into TB buffer (300 mM sodium chloride and 30 mM sodium citrate) and incubating for 15 min at room temperature. Then, after a thorough washing in PBS, the slides were incubated with an avidin-horseradish peroxidase conjugate (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA). The specific labeling of free 3'-OH ends of DNA was visualized by staining the sections with a fast 3,3'-diaminobenzidine tetrahydrochloride tablet (Sigma) dissolved in PBS.

Statistical Analysis. Differences among the treatment groups were assessed by ANOVA using StatSoft statistical software (Tulsa, OK). Differences among the results for the experiments of tumor growth *in vivo* were assessed by ANOVA with a repeated measurement module. $P \leq 0.05$ was considered significant.

RESULTS

***TRAIL* Gene-induced Apoptosis in Cultured Cancer Cells.** In a binary adenoviral vector system, the expression of the *TRAIL* gene in target cells is achieved by cotransfection of Ad/GT-*TRAIL* and the transactivator adenovirus, Ad/PGK-GV16. The expression of the *TRAIL* gene by this system was confirmed *in vitro* in human cancer cell lines by RNase protection assay (Fig. 1A). Treatment of cells with Ad/GT-*TRAIL* + Ad/PGK-GV16 resulted in a strong *TRAIL*-specific band, whereas mock infection or infection with control vectors resulted in undetectable expression. Expression of *TRAIL* on the surface of Ad/GT-*TRAIL* + Ad/PGK-GV16-treated cells was further documented by FACS analysis (Fig. 1B). Whereas no detectable *TRAIL* molecule was found on surfaces of cells treated with PBS or LacZ-expressing vectors, a substantial amount of *TRAIL* was detected on the surfaces of cells treated with the *TRAIL*-expressing vectors. To test whether treatment with the *TRAIL* gene, which is known to elicit apoptosis in a variety of transformed or malignant cells (7, 8), would similarly elicit apoptosis in cultured cancer cells, the cell-killing effect of the *TRAIL* gene was analyzed by quantifying apoptotic cells via flow cytometry or by measuring cell viability via XTT assay. Although the level of apoptosis varied among cell lines, flow cytometry at 48 h after treatment showed a dramatic increase in the number of apoptotic cells in the group treated with Ad/GT-*TRAIL* + Ad/PGK-GV16 in all three of the cell lines tested (Fig. 1C). The results were supported by a cell viability (XTT) study showing a significant difference in cell killing between lines in response to treatment with *TRAIL*-expressing vectors *versus* control vectors (Fig. 1D). Morphological studies using Hoechst nuclear staining revealed nuclear fragmentation (a hallmark of apoptosis) in cells treated with Ad/GT-*TRAIL* + Ad/PGK-GV16 (data not shown). Biochemical studies showed activation of caspase-8 and cleavage of PARP (Fig. 1E). Together, these results demonstrated that treatment with the *TRAIL* gene effectively elicited apoptosis in cultured malignant cells.

Tumor Growth Suppression by Intratumoral Delivery of the *TRAIL* Gene *in Vivo*. To further test the antitumor effect of the *TRAIL* gene, human colon cancer xenografts were established in nude

mice by inoculating DLD-1 cells s.c. into the dorsal flanks of mice as described above in "Materials and Methods." In comparison with treatments with PBS or Ad/GT-LacZ + Ad/PGKGV16, treatment with Ad/GT-*TRAIL* + Ad/PGK-GV16 significantly suppressed tumor growth ($P < 0.001$). Most PBS- or LacZ-treated tumor-bearing mice were killed by day 45 because their tumors had exceeded 1.5 cm in diameter. The growth of the tumors treated with Ad/GT-*TRAIL* was well suppressed until day 40 on average, after which they began to regrow gradually. To test whether regrowing tumors were resistant to the *TRAIL* gene, a second cycle of treatment was initiated in these mice on day 45. As was observed in the first cycle of treatment, tumors were suppressed or moved to regress by the second cycle of treatment, which suggested that the tumor cells remained sensitive to the *TRAIL* gene (Fig. 2A). Histochemical analysis by TUNEL staining 2 days after the first injection of Ad/GT-*TRAIL* revealed extensive apoptosis in tumors (Fig. 2B), which demonstrated that intralesional administration of the *TRAIL* gene effectively killed tumor cells. Furthermore, no observable side effects were found in the mice after the six injections of *TRAIL* vectors, which suggested that such treatment was well tolerated.

Lack of *TRAIL*-induced Toxicity in Normal Cells *in Vitro* and *in Vivo*. To determine whether adenovirus-mediated expression of *TRAIL*, which was reportedly not toxic to normal cells (7, 8), would be toxic to normal cells, the cell-killing effects of *TRAIL* on NHLFs were tested and compared with those of the *Bax* gene by XTT assay. Treatment of NHLFs with the *Bax*-expressing vectors effectively killed the cells (Fig. 3A), consistent with our previous finding that overexpression of the *Bax* gene caused apoptosis in normal cells (11). In contrast, treatment of NHLFs with the *TRAIL*-expressing vectors resulted in the same cell viability curve as that of the vector control group, which suggested that overexpression of the *TRAIL* gene was nontoxic to normal cells.

Because human *TRAIL* is cross-reactive to murine cells (1), we also tested the toxicity of the *TRAIL* gene in mice after systemic gene delivery. Adult nude mice (6–8 weeks old) were infused via the tail vein with PBS, Ad/E1-, Ad/GT-LacZ + Ad/PGK-GV16, Ad/GT-*TRAIL* + Ad/PGK-GV16, or Ad/GT-*Bax* + Ad/PGK-GV16 at a total dose of 6×10^{10} particles/mouse. Most mice treated with Ad/GT-*Bax* + Ad/PGK-GV16 became moribund or died within 3 days after vector administration, whereas mice treated with Ad/GT-*TRAIL* + Ad/PGK-GV16 showed no apparent symptoms. Three mice per group were killed on day 4 and day 13 after vector administration. Then liver, spleen, lung, heart, pancreas, kidney, adrenal gland, intestine, gonad, and brain were harvested from these mice for histopathological examination. Except for signs of extramedullary hematopoiesis in the spleen and of scattered ($>2\%$) mitotic or dead hepatocytes in the liver, no other lesions were found in any of the organs examined in mice treated with Ad/GT-*TRAIL* + Ad/PGK-GV16 (Fig. 3B). No difference was found between mice treated with Ad/GT-*TRAIL* + Ad/PGK-GV16 and those treated with control vectors, which suggested that the changes in the spleen and liver of these mice were vector-related rather than *TRAIL* gene-related. In contrast, massive coagulative necrosis was found in the livers of mice treated with Ad/GT-*Bax* + Ad/PGK-GV16. Liver samples from this group showed many hepatocytes that either were dead or exhibited the foregoing alterations (Fig. 3B) and whose nuclei either were missing or had undergone karyorrhexis or karyolysis. This finding is consistent with our previous results that $>60\%$ of hepatocytes underwent apoptosis at 24 h after systemic delivery of Ad/GT-*Bax* + Ad/PGK-GV16 (11). In addition, analysis of serum liver enzymes revealed that the levels of serum AST and serum ALT were normal at all of the time points tested in animals treated with Ad/GT-*TRAIL* + Ad/PGK-GV16 (Fig.

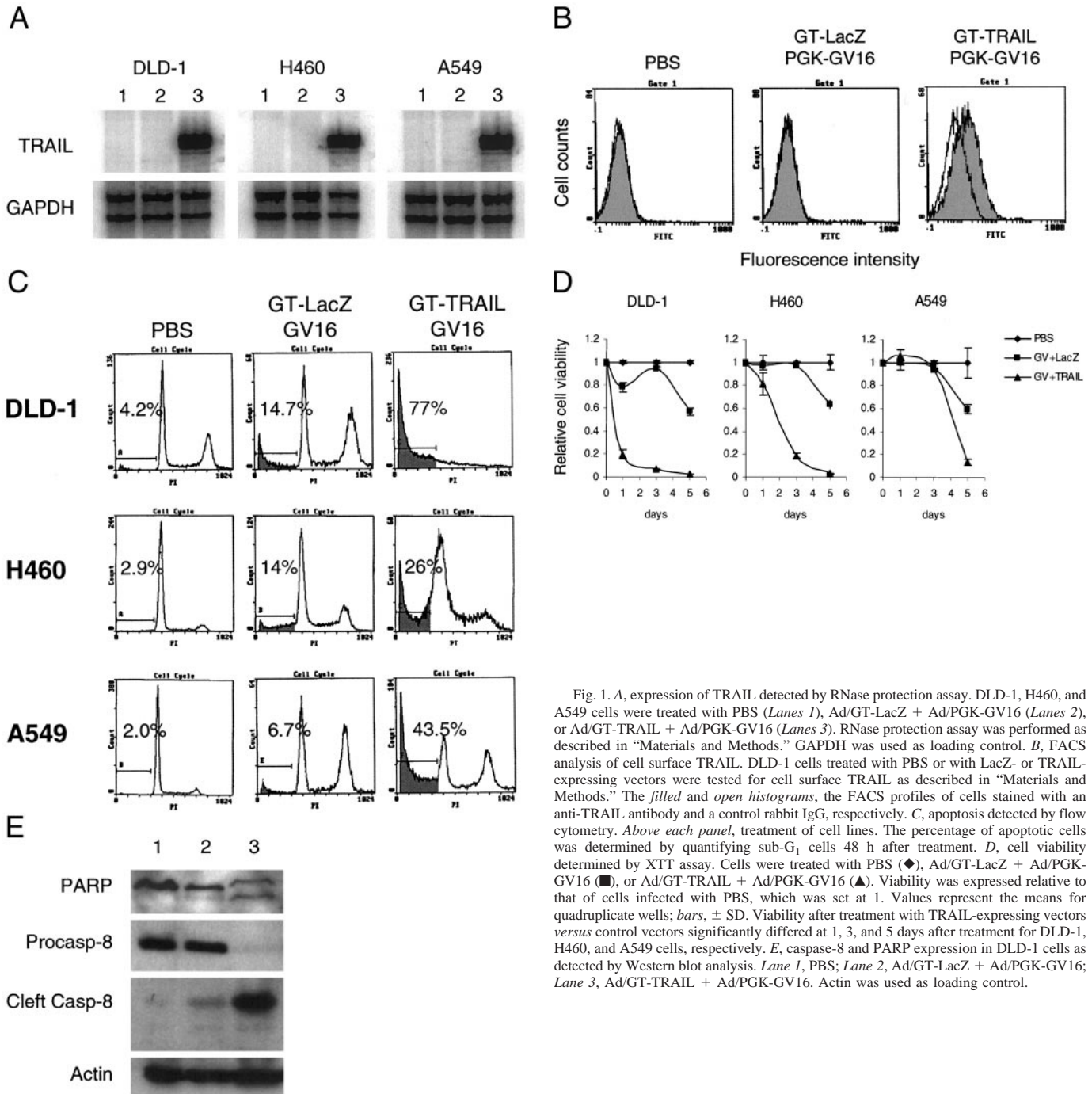


Fig. 1. A, expression of TRAIL detected by RNase protection assay. DLD-1, H460, and A549 cells were treated with PBS (Lanes 1), Ad/GT-LacZ + Ad/PGK-GV16 (Lanes 2), or Ad/GT-TRAIL + Ad/PGK-GV16 (Lanes 3). RNase protection assay was performed as described in "Materials and Methods." GAPDH was used as loading control. B, FACS analysis of cell surface TRAIL. DLD-1 cells treated with PBS or with LacZ- or TRAIL-expressing vectors were tested for cell surface TRAIL as described in "Materials and Methods." The filled and open histograms, the FACS profiles of cells stained with an anti-TRAIL antibody and a control rabbit IgG, respectively. C, apoptosis detected by flow cytometry. Above each panel, treatment of cell lines. The percentage of apoptotic cells was determined by quantifying sub-G₁ cells 48 h after treatment. D, cell viability determined by XTT assay. Cells were treated with PBS (◆), Ad/GT-LacZ + Ad/PGK-GV16 (■), or Ad/GT-TRAIL + Ad/PGK-GV16 (▲). Viability was expressed relative to that of cells infected with PBS, which was set at 1. Values represent the means for quadruplicate wells; bars, ± SD. Viability after treatment with TRAIL-expressing vectors versus control vectors significantly differed at 1, 3, and 5 days after treatment for DLD-1, H460, and A549 cells, respectively. E, caspase-8 and PARP expression in DLD-1 cells as detected by Western blot analysis. Lane 1, PBS; Lane 2, Ad/GT-LacZ + Ad/PGK-GV16; Lane 3, Ad/GT-TRAIL + Ad/PGK-GV16. Actin was used as loading control.

3C). Together, these results suggested that treatment with the TRAIL gene was nontoxic to normal tissues in animals.

Bystander Effect of the TRAIL Gene in DLD-1 Cells. Because TRAIL is believed to elicit apoptosis through interaction with receptors on cell surfaces, it is expected that cancer cells expressing TRAIL would kill their neighboring cells. To test this hypothesis, DLD-1 cells that were infected with adenoviral vector Ad/GT-TRAIL, Ad/GT-Bax, or Ad/GT-LacZ (effector cells) were mixed with DLD-1 cells that were infected with Ad/CMV-GFP (target cells) at a ratio of 1:1; the mixed culture was then seeded in 6-well plates and infected with Ad/PGK-GV16 to induce expression of the TRAIL, Bax, and LacZ genes. The induction of TRAIL and Bax gene expression after mixed culture allowed the effector cells to survive until they were in contact

with the target cells. Forty-eight h after the mixed culture and induction of transgenes, dead cells were labeled with PI and subjected to flow cytometric analysis to determine the extent of cell death in both the effector- and target-cell populations. Coculture of TRAIL-expressing and GFP-expressing cells resulted in the death of >90% of both effector and target cells (Fig. 4A). In contrast, coculture of Bax-expressing and GFP-expressing cells resulted in massive death of the former but not the latter cells, whereas coculture of LacZ- and GFP-expressing cells resulted in only background levels of death in both cell types. These results suggested that overexpression of the TRAIL gene killed both the transduced cells and their neighbors, whereas overexpression of the Bax gene killed transduced cells only.

To test whether TRAIL-expressing DLD-1 cells exert such by-

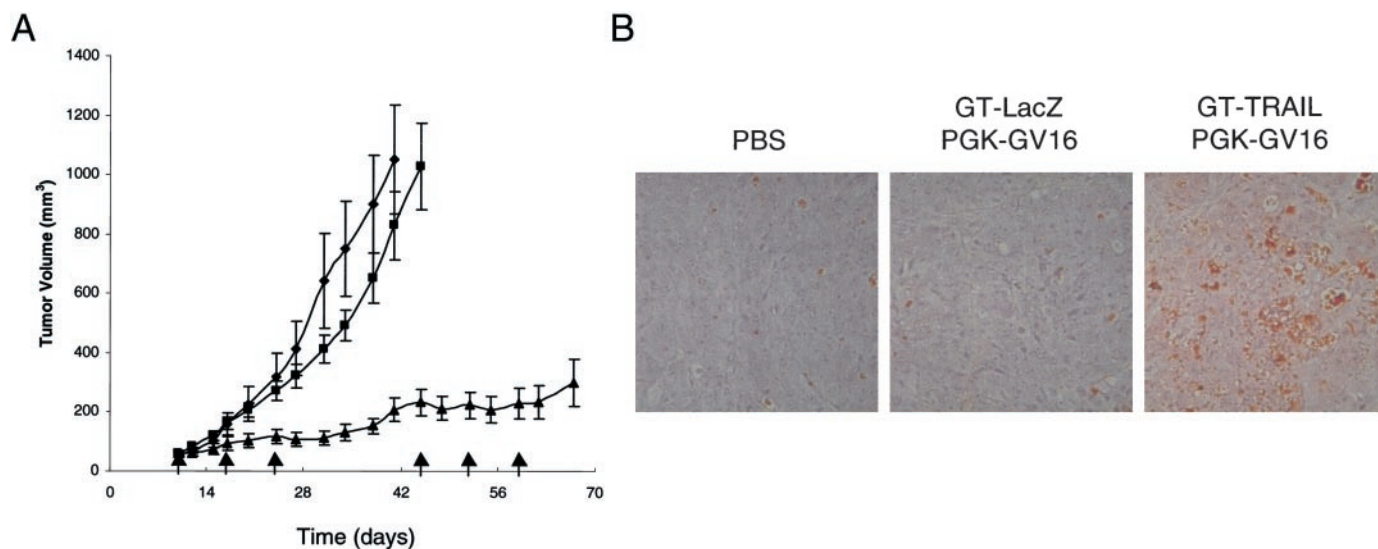


Fig. 2. A, tumor growth in s.c. xenograft model derived from DLD-1 cells. Tumor volume was monitored over time (days) after inoculation of tumor cells. ◆, PBS; ■, Ad/GT-LacZ + Ad/PGK-GV16; ▲, Ad/GT-TRAIL + Ad/PGK-GV16. Arrow, time point at which treatment was given. Values represent the means for 10 animals per group; bars, ± SE. Tumor growth was significantly suppressed in mice treated with TRAIL-expressing vectors versus control vectors ($P < 0.001$). B, *in situ* TUNEL staining in DLD-1 tumors at 2 days after first dose of vectors. Above each panel, the treatment.

stander effect on neighboring normal cells, GFP-expressing NHLF cells were used as target cells. Coculture of LacZ-, Bax- or TRAIL-expressing DLD-1 cells with GFP-expressing NHLF cells did not cause cell death of GFP⁺-cells (Fig. 4B), which suggested that there was no bystander effect on NHLF from TRAIL-expressing DLD-1 cells. To test whether the TRAIL gene expressed in normal cells would cause bystander effects on malignant susceptible cells, LacZ-, Bax-

and TRAIL-expressing NHLF cells were cocultured with GFP-expressing DLD-1. Survival of GFP⁺ cells was then determined by flow cytometry analysis at 48 h after mixed culture. Whereas coculture of LacZ- or Bax-expressing NHLF with GFP-expressing DLD-1 cells had no effect on the survival of DLD-1 cells, coculture of the TRAIL-expressing NHLF with GFP-expressing DLD-1 cells reduced surviving GFP⁺ cells to less than 50% (Fig. 4C), which suggest that

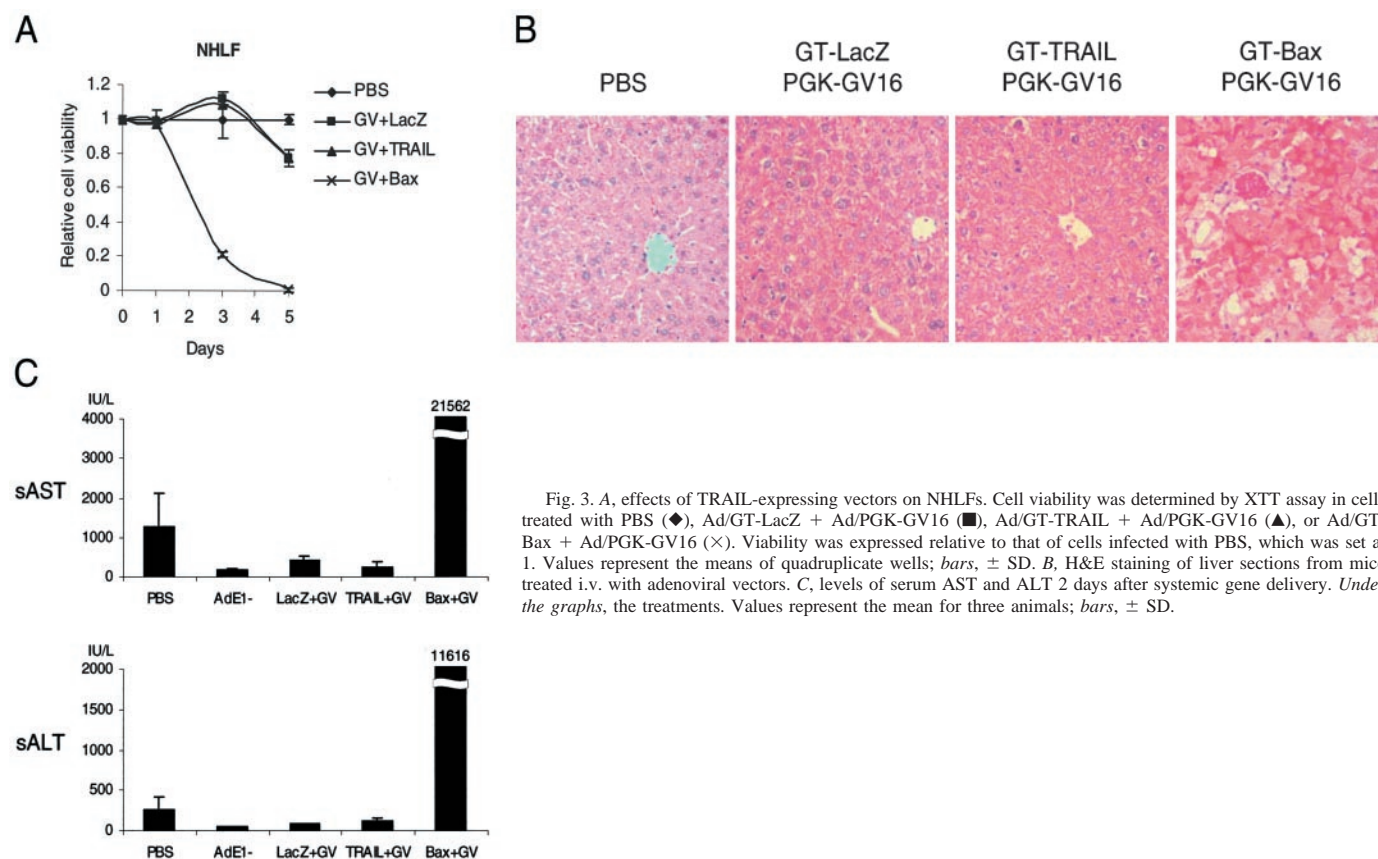


Fig. 3. A, effects of TRAIL-expressing vectors on NHLFs. Cell viability was determined by XTT assay in cells treated with PBS (◆), Ad/GT-LacZ + Ad/PGK-GV16 (■), Ad/GT-TRAIL + Ad/PGK-GV16 (▲), or Ad/GT-Bax + Ad/PGK-GV16 (×). Viability was expressed relative to that of cells infected with PBS, which was set at 1. Values represent the means of quadruplicate wells; bars, ± SD. B, H&E staining of liver sections from mice treated i.v. with adenoviral vectors. C, levels of serum AST and ALT 2 days after systemic gene delivery. Under the graphs, the treatments. Values represent the mean for three animals; bars, ± SD.

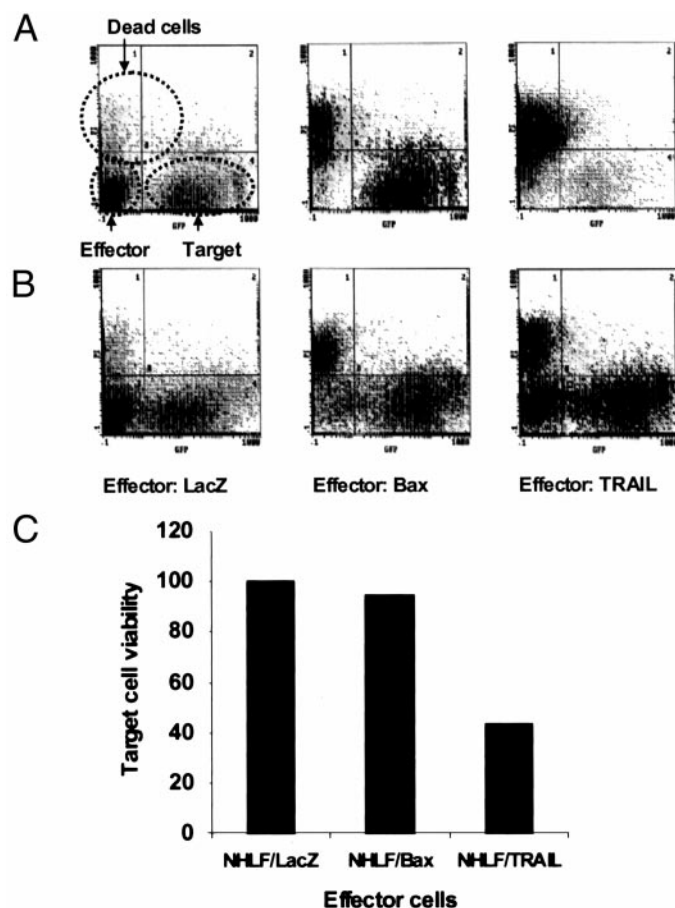


Fig. 4. Bystander effects of the *TRAIL* gene. Ad/GT-*TRAIL*-, Ad/GT-*Bax*-, or Ad/GT-*LacZ*-transfected cells (*Effector*) were cocultured with Ad/CMV-GFP-transfected cells (*Target*). Expression of *TRAIL*, *Bax*, or *LacZ* was induced by infection of cells with Ad/PGK-GV16. Cells were then stained with PI to identify dead cells, which were then sorted to identify the PI-positive fraction by flow cytometry. A, DLD-1 cells were used as both effector and target. B, DLD-1 cells were used as effector and NHLF cells as target. C, NHLF cells were used as effector and DLD-1 cells as target. Bar graph, percentage of living target DLD-1 cells. Values represent an average of the results obtained from two independent experiments.

the *TRAIL* gene that is expressed in normal cells can elicit a bystander effect in susceptible neighboring malignant cells, although the normal transduced cells themselves are not affected.

Visualized Bystander Effects of the *TRAIL* Gene in Cancer Cells. To further document the bystander effects of the *TRAIL* gene on cancer cells, two fusion genes, one encoding a chimera protein consisting of GFP (18) and *TRAIL* and the other consisting of GFP and *Bik* [a member of the *Bcl-2* family proteins that promotes apoptosis through the mitochondrial pathway (15, 19)], were used to visualize transduced and nontransduced cells under a fluorescent microscope. Human colon cancer DLD-1 and Lovo cells, human lung cancer A549 cells, and human embryonic kidney 293 cells were transfected with plasmids expressing GFP-*TRAIL* (pEGFP-*TRAIL*) or GFP-*Bik* (pEGFP-*Bik*). Cells transfected with plasmid expressing wild-type GFP (pEGFP-C3) were used as a control. Cells were then observed under a fluorescent microscope at 24 h after transfection. Cells that were transfected with the GFP-*Bik* did not show any normal morphology, whereas cells transfected with wild-type GFP showed a uniform green staining. In contrast, cells that were transfected with the GFP-*TRAIL* construct were green, mainly in their cytoplasm, which indicated that, like *TRAIL*, GFP-*TRAIL* probably moved to the cytoplasmic membrane (1, 2). In all of the cell lines tested, transfection with pEGFP did not change the morphology of cells (Fig. 5A),

whereas transfection with pEGFP-*Bik* resulted in a typical morphological change of dying cells (Fig. 5A). pEGFP-*Bik*-transfected cells rounded up, shrunk, and become detached from the bottom of culture dishes and, in some cases, were broken down into GFP-positive debris. Nevertheless, the neighbors of these pEGFP-*Bik*-transfected cells showed a normal morphology, which suggested that cells expressing the *Bik* gene did not have bystander effects. Cells transfected with pEGFP-*TRAIL* also showed a morphology typical of dying or dead cells (Fig. 5A), but in each case the transfected (GFP-positive) cell was surrounded by other cells showing the same morphology. This suggested that the *TRAIL* gene product possessed bystander effects. Moreover, in most cases, all of the neighboring cells were killed and formed a microplaque under microscope.

Effects of GFP-*TRAIL* and GFP-*Bik* Genes on Normal Cells.

To test whether the GFP-*TRAIL* and GFP-*Bik* genes could also cause cell death in normal cells, pEGFP-*TRAIL*, pEGFP-*Bik*, and pEGFP-C3 (control) were transfected into NHBEs and NHLFs. Morphological changes in the transfected cells were then examined 24 h after the transfection, as described above. This revealed that GFP was similarly distributed within normal cells as cancer cells. In cells transfected with pEGFP-C3 and pEGFP-*Bik*, GFP was distributed uniformly within cells, whereas in cells transfected with pEGFP-*TRAIL*, GFP localized in the cytoplasm. Transfection with pEGFP-*Bik* killed both NHBE cells and NHLFs (Fig. 5B). In contrast, transfection with pEGFP-C3 or pEGFP-*TRAIL* apparently had no cytopathic effect on these cells (Fig. 5B). Moreover, cells neighboring the transfected cells showed no signs of the bystander effect, thus suggesting that the *TRAIL* gene was not toxic to NHBE cells and NHLFs.

The Proapoptotic and Bystander Effects of the *TRAIL* Gene Was Not Transferable with Medium.

As a type II membrane protein, *TRAIL* can be cleaved from the cell membrane to form a soluble molecule (1, 2). Both cell-surface-expressed *TRAIL* and soluble *TRAIL* rapidly induce apoptosis in a wide variety of transformed cell lines via interaction with the death receptors (3, 4). To test whether the cell-killing effect was mediated by soluble factors, media from the *TRAIL*-, *Bax*-, or *LacZ*-expressing DLD1, NHLF and A549 cells were collected and added to fresh DLD-1 cells. Medium from untreated DLD1, NHLF, or A549 cells was used as control. The cell killing effects of these media were then monitored over time for up to 72 h by XTT assay. As a result, no significant cytotoxicity (in terms of cell killing or cell growth) was found (Fig. 6). In addition, soluble *TRAIL* in medium samples from *TRAIL*-, *Bax*-, or *LacZ*-expressing NHLF cells was assayed using ELISA and commercially available antibodies that were able to detect 6.25 ng/ml of soluble *TRAIL*. In all of the samples tested, no *TRAIL* was detected (data not shown). Together, these results suggested that secretion of soluble *TRAIL* from the *TRAIL*-expressing cells is not substantial and that the cell-killing effect of soluble factors, if any, is minimal.

DISCUSSION

TRAIL has become an attractive molecule for the treatment of cancers because it specifically kills tumor cells (7, 8). Studies of recombinant *TRAIL* protein have revealed that the extracellular portion of the *TRAIL* molecule is sufficient for its antitumoral activity but that homotrimerization is necessary for the *TRAIL* protein to retain this activity (7, 8), which suggests that the conformational structure of *TRAIL* is crucial for its interaction with its receptors. In the study reported here, we asked whether introducing the *TRAIL* gene directly into tumor cells would result in the expression of biologically active molecules that could effectively kill malignant cells *in vitro* and *in vivo*. As our results clearly demonstrate, they could and did.

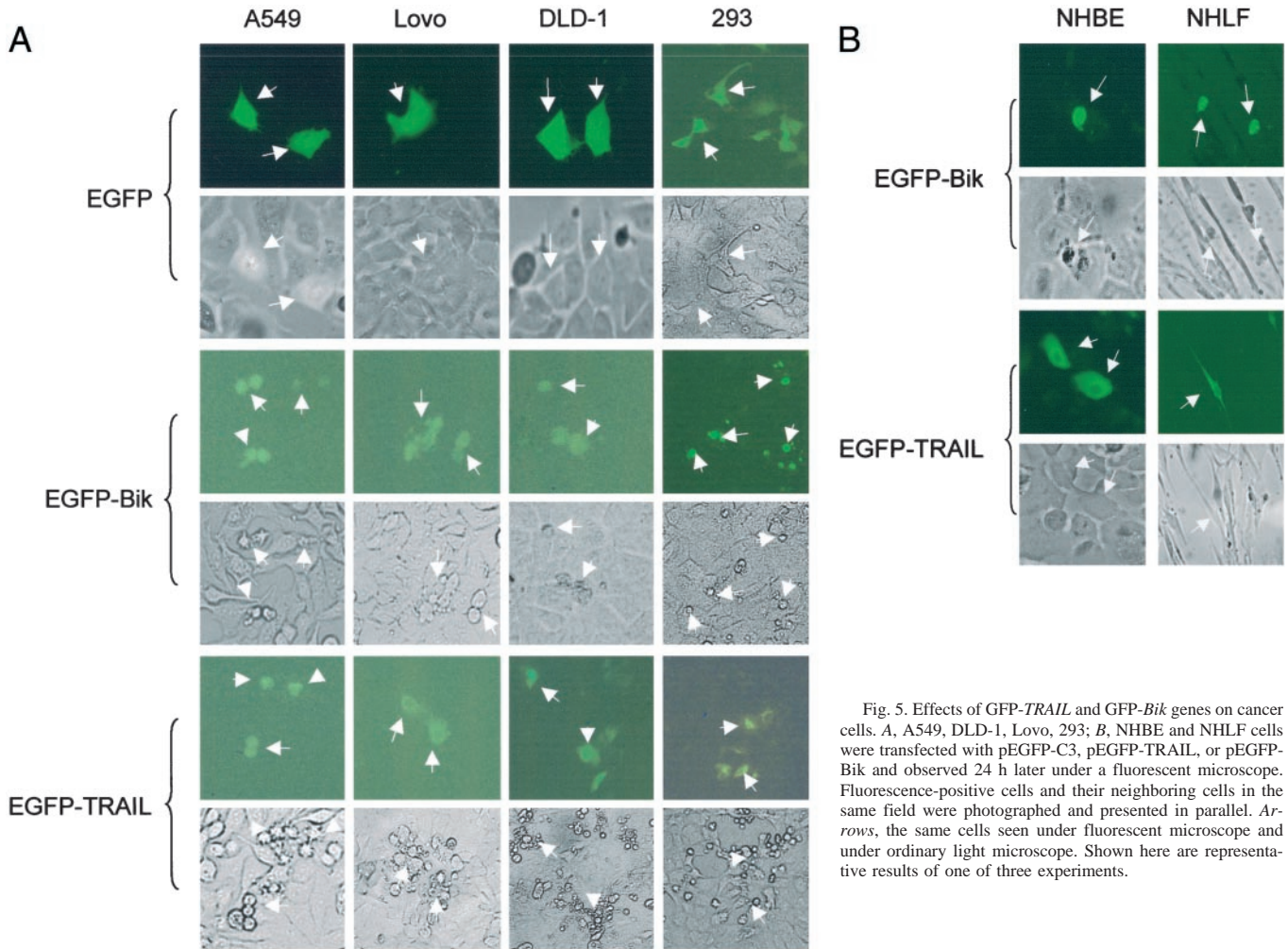


Fig. 5. Effects of GFP-TRAIL and GFP-Bik genes on cancer cells. A, A549, DLD-1, Lovo, 293; B, NHBE and NHLF cells were transfected with pEGFP-C3, pEGFP-TRAIL, or pEGFP-Bik and observed 24 h later under a fluorescent microscope. Fluorescence-positive cells and their neighboring cells in the same field were photographed and presented in parallel. Arrows, the same cells seen under fluorescent microscope and under ordinary light microscope. Shown here are representative results of one of three experiments.

TRAIL demonstrates antitumor activity in a variety of tumors (1, 2, 7), although different tumors may vary in their sensitivity to it. This phenomenon was observed in our study. Of the three tumor cell lines tested, DLD-1 and H460 were highly sensitive and A549 was relatively less sensitive to TRAIL gene therapy. The mechanisms of this differential sensitivity of malignant cells to TRAIL treatment, as well as of the differential cell killing of normal cells versus malignant cells, remain to be delineated. They can be partially explained by the presence of multiple receptors for TRAIL that function as either

death-inducing or decoy receptors (6, 20, 21). Some groups have proposed that expression of decoy receptors may confer resistance to normal tissues (20, 21); others have suggested that levels of the intracellular caspase/apoptosis inhibitors including FLIP and Bcl-X_L may make cells resistant (22–24). However, using RNase protection assay, we have found that the different sensitivities among the three cell lines tested in this study could not be explained by the levels of DR4, DR5, and DcR1 in these cells (data not shown). This finding has also been reported by others (10, 22, 23). Interestingly, there are also reports that treatment with certain chemotherapy reagents or with ionizing radiation can sensitize resistant cells to TRAIL-induced apoptosis (7, 9, 25). This suggests in turn that the combination of chemotherapy with TRAIL gene therapy may increase the efficacy of anticancer treatment. However, one of these reports also mentioned that normal cells could be sensitized to TRAIL-induced apoptosis (10). This suggests that such a combination treatment may also increase toxicity. Nevertheless, such an increase in toxicity in response to this combination therapy may be avoided if TRAIL expression can be limited locally to tumors.

Moreover, our study revealed that treatment with the TRAIL gene is nontoxic to *in vitro* cultured normal human cells and *in vivo* in animals. These results are consistent with previous reports by others who used recombinant TRAIL proteins (7, 8). However, some immortalized nontransformed cells have been reported to be sensitive to TRAIL-induced cell death (10). In our own study, we also found 293, a human adenovirus 5-transformed human embryonic kidney cell line

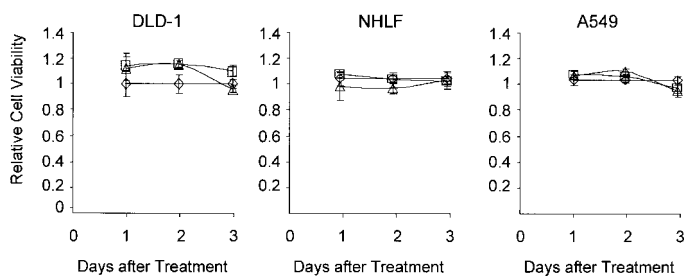


Fig. 6. Effects of medium from TRAIL-expressing cell cultures. At 48 h after treatment with adenoviral vectors, media were collected from cultures of DLD-1, NHLF, and A549 cell cultures cells expressing TRAIL, Bax, or LacZ. Medium from mock-infected DLD-1 cells was used as control. These media were added to freshly cultured DLD-1 cells, and cell viability was measured by XTT assay in cells treated with medium from untreated cells (◇), Ad/GT-LacZ + Ad/PGK-GV16-treated cells (□), or Ad/GT-TRAIL + Ad/GV16-treated cells (△). Viability was expressed relative to that of cells treated with culture medium from untreated cells, which was set at 1. Values represent the means for quadruplicate wells; bars, ± SD. P ≤ 0.14 among groups.

(26) was highly sensitive to TRAIL-expressing plasmid. More recently, it has been reported that human hepatocytes are very sensitive to TRAIL-induced apoptosis (27), which suggests that results obtained from animal studies may not be directly applied to humans. Whereas observation of toxic effects of TRAIL on human hepatocytes raised concern about the potential toxicity of TRAIL when administered systemically, toxicity may be reduced by vector-targeting strategies.

Theoretically, treatment with the TRAIL may elicit bystander effects either through interaction of cell surface TRAIL molecules with receptors on neighboring cells or through the action of soluble TRAIL from the TRAIL-expressing cells. In the present study, we have demonstrated the ability of the *TRAIL* gene to exert an apoptotic bystander effect on cancer cells. Using adenovirus- or plasmid-mediated gene transfer, we compared the cell killing effects of the *TRAIL* gene with that of the *Bax* and *Bik* genes (15, 28). Both *Bik* and *Bax* are members of the Bcl-2 gene family and belong to a group of death agonists that can induce apoptosis via mitochondrial pathways in a variety of cells (19). In the present study, overexpression of the *Bax* or *Bik* gene induced apoptosis in both normal and cancer cells, but with no apparent bystander effects. In contrast, overexpression of *TRAIL* induced apoptosis and bystander effects in susceptible malignant cells but not in normal cells. These results suggest that the bystander effect of the *TRAIL* gene was not caused by the nonspecific effects of apoptotic cells nor by the transfection methods.

As a type II membranous protein, there is speculation that membrane-bound *TRAIL* can be cleaved by a protease and turned into a soluble form that exerts an apoptotic reaction. Thus, soluble TRAILS, cleaved from TRAIL-expressing cells, may function as cytokines and act on remote cells, resulting in antitumor or side effects. Nevertheless, in this study, we have found a substantial amount of TRAIL was present on the surface of the cells treated with the TRAIL-expressing vectors. However, no detectable soluble TRAIL was found in the medium of the TRAIL-expressing cell cultures. Furthermore, the cell-killing effect of the *TRAIL* gene was not transferable with the medium of TRAIL-expressing cell cultures. These results collectively suggest that the proapoptotic activity of the *TRAIL* gene is mainly elicited via membrane-bound TRAIL, and that soluble factors contribute little to antitumor activity and to the bystander effect of the *TRAIL* gene. One possible explanation is that the effects of soluble TRAIL may be dose-dependent, conformation-dependent, or both. It has been reported that trimerized recombinant TRAIL is more effective than the monomer form; thus, conformational differences in soluble TRAIL proteins may also dramatically affect their activities (29). Although the effects of recombinant soluble TRAIL on susceptible cells have been documented (1, 2, 7, 8), it is possible that these effects were elicited with high doses of TRAIL that would not be achievable by spontaneous cleavage of TRAIL from cultured mammalian cells. The fact that no detectable TRAIL was found in any of the media of the TRAIL-expressing cell cultures suggested that spontaneous cleavage of the TRAIL is minimal. In fact, the *TRAIL* gene is constitutively expressed in a variety of normal tissues. Yet, under physiological conditions, the release of soluble TRAIL from such TRAIL-expressing tissues is not substantial enough to cause liver damage, although normal primary human hepatocytes are susceptible to soluble TRAIL (27). Although the underlying mechanisms remain to be characterized, our results suggest that the TRAIL gene therapy may not necessarily cause substantial liver toxicity in humans, as long as the *TRAIL* gene is not expressed in cells adjacent to hepatocytes. On the other hand, malignant cells distant from TRAIL-transfected cells may also be unaffected. Therefore, efficient delivery of the *TRAIL* gene into as many cancer cells as possible is still an important goal in treating cancer patients, although the bystander

effects of the *TRAIL* gene would make the transduction of 100% of cancer cells unnecessary.

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Shunsuke Kagawa, Chao He, Jian Gu, et al.

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