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Eliminating established tumor in nu/nu nude mice by a TRAIL-armed oncolytic adenovirus

Fengqin Dong^{1,2}, Li Wang^{1,3}, John J. Davis^{1,3}, Wenxian Hu¹, Lidong Zhang¹, Wei Guo¹, Fuminori Teraishi¹, Lin Ji¹, and Bingliang Fang^{1,*}

¹ Department of Thoracic and Cardiovascular Surgery, Unit 445, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030, USA.

² Department of Metabolism and Endocrinology, The First Affiliated Hospital, Zhejiang University, 79 Qingchun Road, Hangzhou, Zhejiang, 310003, People's Republic of China.

Abstract

Purpose—The tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL) and oncolytic viruses have recently been investigated extensively for cancer therapy. However, preclinical and clinical studies have revealed that their clinical application is hampered by either weak anticancer activity or systemic toxicity. We examined whether the weaknesses of the two strategies can be overcome by integrating the *TRAIL* gene into an oncolytic vector.

Experimental Design—We constructed a TRAIL-expressing oncolytic adenovector designated Ad/TRAIL-E1. The expression of both the *TRAIL* and viral *E1A* genes is under the control of a synthetic promoter consisting of sequences from the human telomerase reverse transcriptase promoter and a minimal cytomegalovirus early promoter. The transgene expression, apoptosis induction, viral replication, antitumor activity and toxicity of Ad/TRAIL-E1 were determined *in vitro* and *in vivo* in comparison with control vectors.

Results—Ad/TRAIL-E1 elicited enhanced viral replication and/or stronger oncolytic effect *in vitro* in various human cancer cell lines than a TRAIL-expressing replication-defective adenovector or an oncolytic adenovector expressing green fluorescent protein. Intralesional administration of Ad/TRAIL-E1 eliminated all subcutaneous xenograft tumors established from a human non-small cell lung cancer cell line, H1299, on nu/nu nude mice, resulting in long-term tumor-free survival. Furthermore, we found no treatment-related toxicity.

Conclusions—Viral replication and antitumor activity of oncolytic adenovirus can be enhanced by the *TRAIL* gene and Ad/TRAIL-E1 could become a potent therapeutic agent for cancer therapy.

Keywords

lung neoplasma; apoptosis; virotherapy; death ligand

Introduction

The tumor necrosis factor- α -related apoptosis-inducing ligand (TRAIL), which induces tumor-selective apoptosis via death receptor-mediated apoptosis, is regarded as a promising anticancer agent. However, the clinical application of recombinant TRAIL may be limited by the facts that only large amounts of TRAIL elicit antitumor activity (1–4) and that a substantial number of tumor cells are resistant to recombinant TRAIL proteins (5;6). On the other hand,

*Correspondence: Bingliang Fang; E-mail: bfang@mdanderson.org; Telephone: 713-563-9147; Fax: 713-794-4901.

³These authors contributed equally to this work.

virotherapy with oncolytic viruses is designed to selectively replicate and kill tumor cells by using viral mutants (7–9) or by using tumor-specific promoters (10;11) to control viral replication. More than 10 clinical trials involving more than 200 patients with cancer have been conducted with Onyx-015, the first oncolytic virus described (12–14). The reported results showed that oncolytic viruses administered intratumorally, intraperitoneally, and intravenously were well tolerated. However, the efficacy of Onyx-015 as a single agent is also limited, possibly because of inefficient cell lysis and release of their progeny, which may be defective in cancer cells (15). Indeed, a clinical trial with Onyx-015 vector showed that even though the vector was present and replicating in tumors as long as a week after its intralesional injection, no obvious tumor necrosis or apoptosis was detected (16), suggesting that its replication alone may be insufficient to induce cell death.

We hypothesized that integrating *TRAIL* gene therapy with an oncolytic adenovector will overcome the weaknesses of the *TRAIL* therapy and virotherapy used individually. An oncolytic virus will replicate in tumor cells, thereby increasing the copy number or expression levels of the human telomerase reverse transcriptase (hTERT)-*TRAIL* gene, and the expression of *TRAIL* will lead to apoptosis, facilitating lysis of tumor cells, release of vector progeny, and spread of the vector within tumors. Moreover, the bystander effect of the *TRAIL* gene will enable the killing of untransduced cells in tumor tissue. Furthermore, we have recently found that a synthetic promoter consisting of sequences from the hTERT promoter and a minimal cytomegalovirus (CMV) early promoter (designated hTC promoter) has stronger transcriptional activity than the wild-type hTERT promoter has and retains the tumor selectivity of the hTERT promoter. A green fluorescent protein (GFP)-expressing oncolytic virus, Ad/hTC-GFP-E1, whose transgenes, GFP and E1A, are both under the control of the hTC promoter, induced tumor-specific transgene expression and oncolysis (17). Thus, an hTC-driven *TRAIL*-expressing oncolytic adenovector may have potent antitumor activity with minimal toxicity to normal cells.

To test whether the therapeutic potency of the *TRAIL*-armed oncolytic virus is greater than that of an oncolytic virus alone, we constructed the adenovector Ad/*TRAIL*-E1 and compared its antitumor activity with that of a *TRAIL*-expressing replication-defective adenovector or and that of the GFP-expressing oncolytic adenovector both *in vitro* and *in vivo*. Our results showed that integrating the three components (the *TRAIL* gene, oncolytic virus, and hTERT promoter) into a single therapeutic agent effectively induced potent tumor-specific cytotoxic effects.

Materials and methods

Cell lines and cell culture

293 cells, human NSCLC cell lines A549, H1299, H460 and the human colon cancer cell line DLD1 and its *TRAIL*-resistant derivative, DLD1/*TRAIL*-R, were maintained in our laboratory. The A549 cells were cultured in Ham's/F12 medium containing 10% heat-inactivated fetal bovine serum (FBS) and 1% (v/v) antibiotics (penicillin and streptomycin) (Life Technologies, Rockville, MD). The other cell lines were maintained in RPMI 1640 medium containing 10% (v/v) heat-inactivated FBS and 1% (v/v) antibiotics. The NHFBs were maintained in DMEM containing 10% heat-inactivated FBS and 1% antibiotics. All cells were cultured at 37°C in a humidified incubator containing 5% CO₂.

Vector production

The hTC promoter was created as described previously (17). Ad/CMV-GFP, Ad/g*TRAIL*, Ad/GFP-E1, and Ad/*TRAIL*-RGD have been described previously (18;19). The recombinant oncolytic vector Ad/*TRAIL*-E1 was constructed by placing hTC upstream of both the *TRAIL*

expression cassette and the *E1A* gene in a shuttle plasmid designated pAd/hTC-TRAIL-E1. The plasmid was then transfected with a *Cla*I-digested 35-kb fragment of adenovirus type 5 into 293 cells, and Ad/TRAIL-E1 was identified by PCR analysis. The vector was plaque-purified twice, expanded in 293K cells, and purified by two cycles of cesium chloride banding.

The sequences in the hTC-*TRAIL* cassette and the hTC-*E1* region were verified by DNA sequencing. Vector amplification, purification, titration, and quality tests were performed as previously reported (20). The titer used in this study was determined by the absorbancy of the dissociated virus at $A_{260\text{nm}}$ (1 $A_{260\text{nm}}$ unit = 10^{12} vp/mL), and the titers determined with a plaque assay were used to determine additive information. Particle:infectious unit ratios were usually between 30:1 and 100:1. Thus, the MOI of 100 vp was equivalent to an MOI of 1 to 3 infectious units. Unless otherwise specified, Ad/CMV-GFP was used as the vector control, and PBS was used as the mock control.

RT-PCR analysis

Total RNA was isolated by using Trizol (Invitrogen) according to the manufacturer's instructions, mixed with a random hexamer primer, and incubated at 70°C for 5 min. Single-strand cDNA was synthesized from 0.5 µg of total RNA by using Moloney murine leukemia virus reverse transcriptase and deoxynucleoside triphosphate at 42°C for 1 h and 90°C for 5 min; synthesis was stopped at 4°C. The mRNAs for TRAIL were amplified by PCR with specific primers. The sequences of the primers for TRAIL were 5'-AGTCCATATTCTGCATCTTT and 5'-AGATGACAGTTATTGGGACC. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control.

The PCR reaction was carried out as follows: 1× at 94°C for 3 min; 35× at 94°C for 45 sec, 55°C for 45 sec, and 72°C for 1 min; and 1× at 72°C for 10 min. PCR products were analyzed by using agarose gel electrophoresis and visualized with the use of ethidium bromide.

Western blot analysis

To assess the protein concentrations in the cell extracts, we performed Western blotting. After being washed with PBS, the four NSCLC cells lines were lysed in triple-detergent lysis buffer (20-mM Tris-Cl, pH 8.0, 150-mM sodium chloride, 0.02% sodium azide, 0.1% SDS, 100-µg/mL phenylmethylsulfonyl fluoride, 1 µg/mL aprotinin, 1% Nonidet P-40, 0.05% sodium deoxycholate, and complete proteinase inhibitors). Lysates were spun down at 15,000 rpm for 15 min at 4°C, and the supernatant was carefully collected. The protein concentration was assessed by the bicinchoninic acid protein assay method (Pierce Chemical Company, Rockford, IL).

The Total extracts (100 µg/lane) were normalized and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE; 12% gels) immunoblot assay, and blotting was performed on a Hybond-electrochemiluminescence membrane (Amersham, Piscataway, NJ). The signal was then detected by electrochemiluminescence solution (Amersham, Arlington Heights, IL). Western blot analyses were performed using specific antibodies against caspase-8 (Medical & Biological Laboratories CO., Woburn, MA), E1A and caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (Sigma, St. Louis, MO), as described previously (21).

Finally, horizontal scanning densitometry was performed on Western blots using Adobe Photoshop software (Adobe Systems, Inc.). The expression of β-actin was used as an internal control.

Crystal violet staining assay

Cells were seeded into 12-well plates and infected with different doses of the viruses. After 48 h, the culture medium was removed by inverting the plates and shaking out the medium. Then, to fix the cells, 500 μ L of 1% glutaraldehyde in PBS was added to each well for 15 min at room temperature. After the 1% glutaraldehyde in PBS was removed by inverting and shaking the plates, 500 μ L of 0.5% (w/v) crystal violet was added to each well for 15 min. Finally, the plates were washed under gentle tap water until clear and allowed to air dry.

Cell viability assay

The viability of the cell lines was determined by SRB colorimetric assay, as previously described (22). Briefly, after fixation of adherent cells with trichloroacetic acid in a 96-well microplate, the protein was stained with SRB, and the optical density was determined at 570 nm to reflect the number of stained cells, representing cell viability. The percentage of viable cells was determined relative to the cell viability of the PBS control, which was considered 100%. Each experiment was performed in quadruplicate and repeated at least three times.

Flow cytometric assay

We used flow cytometry assay to determine the TRAIL gene expression and apoptosis. For apoptosis assay, both floating and attached cells were collected and washed twice with cold PBS. The cells were then fixed with cold 70% ethanol and kept overnight at 4°C. Thirty minutes before the flow cytometric assay, we performed propidium iodide (PI) staining (1 mL PI, 10 μ L RNase, and 9 mL PBS for a final concentration of 50 μ g/mL. The flow cytometric evaluation of cell cycle status and apoptosis was performed as previously described (18;19). The percentages of cells in the sub-G₁, G₁, S, G₂, and M phases were calculated using Cell Quest software (Becton-Dickinson, San Jose, CA). FACS analysis for TRAIL expression on cell surface was performed as described previously (23). In brief, 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 fluorescein isothiocyanate (FITC)-labeled goat anti-rabbit Ig antibody (Pharmingen, San Diego, CA) 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.

Adenovirus replication assay

To assess the efficacy of adenovirus replication in the various cell lines, the cells were infected with adenovectors at the doses described in the text. Four hours after their infection, the cells were washed with PBS three times to remove any free vector in the medium. The cells were then cultured in fresh medium for various times as indicated in the text. They were then harvested and lysed, and the viral vector replication in cell lysate was determined using the tissue culture infectious dose 50 (TCID₅₀) assay in fresh 293 cells as described previously (24).

Animal study

Six-week-old female specific pathogen-free nu/nu nude mice were purchased from Charles River Laboratories Inc. (Wilmington, MA) and housed at The University of Texas M. D. Anderson Cancer Center under conventional conditions. Animal experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (National Institute of Health Publication number 85-23) and the institutional guidelines of M. D. Anderson Cancer Center.

We injected 2×10^6 H1299 cells in 100 μ L of PBS subcutaneously into the dorsal flanks of mice using a 25-gauge needle. One week after the tumor cell inoculation, when the tumors had grown to 3–5 mm in diameter, the mice were randomized into five treatment groups: PBS, Ad/CMV-GFP, Ad/GFP-E1, Ad/TRAIL-RGD, and Ad/TRAIL-E1. For each treatment, 100 μ L of PBS with or without 2.5×10^{10} vp was injected intratumorally with a 27-gauge needle. The treatments were repeated every 3 days for a total of four treatments.

The tumors were measured every 3 days with calipers, and their volumes were calculated using the formula: $a(b^2) \div 2$, for which a and b represented the longest and shortest diameters, respectively. The mice were euthanized when their tumors reached 1.5 cm in diameter or became ulcerated.

To assess the toxicity of the treatments, we collected blood samples 5 days after the final injection by cutting the tips of the tails and measured the concentrations of serum liver enzymes (aspartate aminotransferase and alanine aminotransferase), blood cell counts (red cells and white cells) and hemoglobin levels.

Statistical analyses

Differences between experimental groups were determined by analysis of variance using software from Statistica (Tulsa, OK). A P value of ≤ 0.05 was considered significant.

Results

Construction and characterization of Ad/TRAIL-E1

To construct our TRAIL-armed replication-competent adenoviral vector (Ad/TRAIL-E1), we first constructed a shuttle plasmid-containing expression cassette for both the *TRAIL* and *E1A* genes, phTC-TRAIL-E1 (Figure 1a). Both genes were driven by the hTC promoter. Ad/TRAIL-E1 was constructed by cotransfecting 293 cells with shuttle plasmids and a 35-kb *Cla*I fragment from adenovirus dl324, an E3-deleted type 5 human adenovirus (Figure 1a). The expression of the *TRAIL* gene from Ad/TRAIL-E1 was assessed by using reverse-transcriptase-mediated polymerase chain reaction (RT-PCR) analysis. For this purpose, H1299 human non-small cell lung cancer cells were infected with 1000 multiplicities of infection (MOI) of Ad/CMV-GFP, Ad/GFP-E1, Ad/gTRAIL, Ad/TRAIL-RGD, and Ad/TRAIL-E1. We harvested the cells 12–24 h later and isolated the mRNA for RT-PCR. The results showed that the TRAIL-specific bands were easily detectable in cells treated with Ad/gTRAIL, Ad/TRAIL-RGD, or Ad/TRAIL-E1 but not in other samples (Figure 1b). Moreover, direct PCR analysis from the same mRNA samples yielded no signal, demonstrating that the RT-PCR bands did not result from contamination of vector DNA.

We also tested E1A expression in cancer cells treated with the various vectors. Cells were infected with 100 or 1000 MOI of Ad/CMV-GFP, Ad/TRAIL-RGD, Ad/GFP-E1, or Ad/TRAIL-E1 for 48 h, and cell lysates were analyzed by Western blotting. Treatment with Ad/CMV-GFP and Ad/TRAIL-RGD revealed no detectable E1A expression, whereas treatment with Ad/GFP-E1 or Ad/TRAIL-E1 led to marked expression of E1A (Fig. 1c). The expression of the TRAIL protein on cell surface was determined by FACS analysis as we previously described (23). Increased TRAIL expression was observed in H1299 cells treated with 500 MOI of Ad/TRAIL-RGD and Ad/TRAIL-E1 vectors but not in H1299 cells treated with PBS or the same dose of Ad/CMV-LacZ (Fig. 1d).

Cell killing effect of Ad/TRAIL-E1 in vitro

The oncolytic function of Ad/TRAIL-E1 was evaluated by crystal violet staining in H1299 cells treated with the various adenovectors. Treatment with Ad/TRAIL-E1 at an MOI of 100

viral particles (vp) resulted in complete cell lysis within 48 h. In contrast, treatment of the cells with Ad/GFP-E1 resulted in incomplete cell lysis within 48 h, even at an MOI of 1,000 vp (Figure 2a). These results suggest that Ad/TRAIL-E1 effectively induces oncolysis in some cancer cells.

To further determine the oncolytic effect of Ad/TRAIL-E1, we treated the human lung cancer cell lines H1299, A549 and H460, the TRAIL-sensitive and TRAIL-resistant human colon cancer cell lines DLD1 and DLD1/TRAIL-R, and the normal human fibroblasts (NHFBs) with Ad/TRAIL-E1, Ad/GFP-E1, Ad/TRAIL-RGD, and Ad/CMV-GFP at various MOIs ranging from 30 to 3,000 vp/cell (Figure 2b). Cells treated with phosphate-buffered saline (PBS) were used as a mock control. Cell viability was determined by using a sulforhodamine B (SRB) colorimetric assay 4 days after treatment.

In all the cancer cell lines tested, Ad/TRAIL-E1 effectively elicited oncolytic effects at MOIs of 30 to 300 vp/cell, whereas Ad/GFP-E1 and Ad/TRAIL-RGD induced dramatic oncolytic effects only at MOIs of 300 to 3,000 vp/cell, suggesting that the oncolytic potency of Ad/TRAIL-E1 is at least 10 times greater than that of Ad/TRAIL-RGD or Ad/GFP-E1 (Figure 2b). Treatment with Ad/CMV-GFP induced no detectable oncolytic effect at any of the doses tested or induced only a mild oncolytic effect at higher doses. In the NHFBs, none of the vectors resulted in more than minimal toxicity. Thus, Ad/TRAIL-E1 appears to be highly oncolytic but minimally toxic to normal cells.

Apoptosis induction by Ad/TRAIL-E1 in cancer cells

To determine whether the decrease in cell viability observed after treatment with Ad/TRAIL-E1 was associated with apoptosis, we performed cell cycle analysis using flow cytometry assay. H460, H1299, DLD1, and DLD1/TRAIL-R cells and NHFBs were treated with the various adenovectors at MOIs of 1000 vp per cell for 48 h. The cells were then harvested and analyzed by FACS. We found that treatment with Ad/TRAIL-E1 induced apoptosis in all four cancer cell lines, as evidenced by a marked increase of cells in the sub-G₁ phase relative to the number in the cells treated with Ad/TRAIL-RGD or Ad/GFP-E1 ($P < 0.05$) (Figure 3a), whereas the cancer cells treated with PBS or Ad/CMV-GFP had only background levels of apoptosis (2.0–2.4%). No obvious apoptosis was found in the NHFBs treated with any of the vectors, an observation consistent with the previous one that treatment with these adenovectors led to only minimal loss of cell viability.

Apoptosis induction by Ad/TRAIL-E1 was further confirmed by Western blot analysis of the H460 lung cancer cell line. Treatment with Ad/TRAIL-E1 or Ad/TRAIL-RGD at an MOI of 1,000 for 48 h led to marked cleavage of caspases 3 and 8 (Figure 3b). This cleavage was not detectable, however, in H460 cells treated with Ad/CMV-GFP or Ad/GFP-E1 at the same MOI.

Adenovirus replication in vitro

It is possible that premature TRAIL-mediated apoptosis in TRAIL-sensitive cancer cells blocks replication of the oncolytic TRAIL vector, thereby reducing the oncolytic effects of the TRAIL-armed oncolytic vector. Therefore, we compared the viral replication of Ad/TRAIL-E1 and Ad/GFP-E1 in TRAIL-sensitive DLD1 cells and TRAIL-resistant DLD1/TRAIL-R cells. We treated 1×10^5 DLD1 and DLD1/TRAIL-R cells with Ad/TRAIL-E1, Ad/GFP-E1, Ad/CMV-GFP, or Ad/TRAIL-RGD at MOIs of 100 or 1,000 vp/cell. Four hours after being infected, the cells were washed with PBS three times to remove any free vector in the medium. The cells were then cultured in fresh medium for 48 or 96 h. They were harvested and their lysates analyzed for viral progeny titration in fresh 293 cells by using the median tissue culture infective dose (TCID₅₀) assay. We found 10^6 – 10^8 infectious viral units in DLD1 or DLD1/TRAIL-R cells infected with either Ad/TERT-GFP-E1 or Ad/TERT-TRAIL-E1 (Figure 4a).

No detectable infectious units (ie, $<10^3$, the lowest dilution the assay can measure) were found in the lysates from the cells treated with Ad/CMV-GFP or Ad/TRAIL-RGD, demonstrating that the oncolytic vectors but not the E1-defective vectors were replicating in those cancer cells. An interesting finding was that in both cell lines and at both initial treatment doses, the final yield of infectious viral units in cells treated with Ad/TRAIL-E1 was much higher than it was in those treated with Ad/GFP-E1. A similar result was observed when H1299 cells were treated for up to 7 days with Ad/TRAIL-E1 or Ad/GFP-E1 at an MOI of 500 vp and their viral titer determined in cell lysate (Figure 4b). The significance of this difference between Ad/GFP-E1 and Ad/TRAIL-E1 is not yet clear. However, our results demonstrated that Ad/TRAIL-E1 could effectively replicate in TRAIL-sensitive cancer cells. Nevertheless, in NHFBs, both Ad/TRAIL-E1 and Ad/GFP-E1 produced only background level of viral replication, demonstrating the tumor-specificity of these vectors.

Suppression of tumor growth in vivo

To further evaluate the antitumor activity of Ad/TRAIL-E1, we established human non-small cell lung cancer (NSCLC) xenografts in 6-week-old female nude by inoculating them with H1299 cells. Intratumoral administration of the adenovectors was started when the resulting tumors grew to 3–5 mm in diameter. After 4 intratumoral injection treatments with the vectors, tumor volume was monitored over time.

As we have observed previously (17), treatment of H1299 tumors with Ad/GFP-E1 or Ad/TRAIL-RGD can lead to significant tumor suppression when compared with mock treatment or control vector (Ad/CMV-GFP) treatment ($P<0.05$) (Figure 5A). Nevertheless, tumor growth occurred in a substantial number of animals treated with these two vectors. Although treatment with those two vectors resulted in a tumor-free rate of about 50%, treatment with Ad/TRAIL-E1 led to complete regression of the tumors in all mice. The median survivals in animals treated with PBS or Ad/CMV-GFP were about 31 and 41 days, respectively (Figure 5b). Treatment with either Ad/TRAIL-RGD or Ad/GFP-E1 increased the median survival to about 86 and 87 days. Nevertheless, treatment with Ad/TRAIL-E1 led to tumor-free survival in all animals for 120 days, the longest time point observed in this study, demonstrating that Ad/TRAIL-E1 can effectively elicit antitumor activity *in vivo*. We also evaluated possible treatment-related toxicity by measuring serum liver enzymes and blood cell counts (red blood and white blood cells and platelets) 5 days after the final injection of the treatments. All of the results were within the normal ranges, and no substantial differences were found between the groups (data not shown).

Discussion

The results of our study demonstrated that Ad/TRAIL-E1 was more effective than Ad/TRAIL-RGD or Ad/GFP-E1 at eliciting cytotoxic effects and suppressing tumor growth, supporting our hypothesis that an integrated *TRAIL* gene therapy with an oncolytic adenovector is more effective than *TRAIL* gene therapy or oncolytic adenovirotherapy alone.

The major problem for adenovirus-mediated cancer gene therapy *in vivo* is incomplete transduction of target cancer cells. Whether delivered intratumorally or systemically, adenovectors may not reach every tumor cell. Because of the bystander effect of the *TRAIL* gene (23;25), adjacent cancer cells may be effectively killed by neighboring cells expressing *TRAIL*, but cancer cells beyond the direct contact of *TRAIL*-expressing cells will escape destruction. The use of a replication-competent vector may increase vector spread within the tumor site, thereby dramatically increasing the effective radius of the treatment. An increased therapeutic effect might also result from increased expression of a therapeutic gene that effectively replicates, increasing the number of copies of the viral genome. Moreover, adenovirus E1A is known to induce apoptosis in certain cancer cells by the accumulation of

p53 and activation of procaspase-8 (26;27). Therefore, coordinated triggering of apoptosis by the TRAIL and E1A genes in the oncolytic vector may be another mechanism of enhancing killing of cancer cells. Nevertheless, we found that apoptotic cells determined by subG1 assay were always much lower than actual cell killing determined by cell viability assay. One possible explanation is that oncolytic adenovirus, as reported, can trigger cell death by other mechanisms, such as necrosis and autophagy (28;29). Interestingly, TRAIL can also trigger necrosis or necrosis-like cell death in tumor cells (30;31).

The defects in the lytic cycle and inefficient release of vector progeny may account for the low efficacy of oncolytic virotherapy that has been seen clinically. Incorporating an apoptosis-inducing gene such as *TRAIL* in an oncolytic virovectore will ultimately facilitate lysis of tumor cells, release of vector progeny, and spread of the vector within tumors. However, it is possible that premature TRAIL-mediated apoptosis in TRAIL-sensitive cancer cells blocks replication of the oncolytic *TRAIL* vector. Therefore, we compared the viral replication of Ad/TRAIL-E1 and Ad/GFP-E1 in TRAIL-sensitive DLD1 and TRAIL-resistant DLD/TRAIL-R cells. Both Ad/TRAIL-E1 and Ad/GFP-E1 effectively replicated in both cell lines. This finding was also consistent with our previous observation that 293 cells were susceptible to the full-length *TRAIL* gene (23) but can support the amplification of replication-defective or replication competent *TRAIL*-expressing vectors.

We found it interesting that in both DLD1 and DLD1/TRAIL-R cells and at both initial treatment doses, the final yield of infectious viral units in cells treated with Ad/TRAIL-E1 was much higher than it was in those treated with Ad/GFP-E1. The significance of the difference between Ad/TRAIL-E1 and Ad/GFP-E1 in generating progeny is not yet clear. However, it is known that in addition to inducing apoptosis, TRAIL induces activation of various other signaling pathways, including Akt (32;33), NFκB (32;34), and mitogen-activated protein kinases such as JNK (34;35), p38 (36), and ERK (33;36). Many of those signaling pathways are known to be required for or supportive of viral replication (37–40), yet the roles of these pathways in the replication of oncolytic TRAIL adenovirus are not known.

In summary, we used hTERT promoters with minimal CMV fragments to drive the TRAIL gene and the E1A gene in Ad/TRAIL-E1. Theoretically, this strategy would limit viral replication and TRAIL expression in cancer cells and prevent side effects on normal tissues. As we had expected, Ad/TRAIL-E1 treatment elicited strong cytotoxic effects in NSCLC and colon cancer cell lines and had only minimal effects on cell viability and apoptosis induction in NHFBs. Because various cancer cells, regardless of their tissue origin, have high hTERT promoter activity (41;42) and are susceptible to TRAIL-mediated apoptosis (2), we believe that Ad/TRAIL-E1 may be a promising potent, broad-spectrum therapeutic agent against cancer.

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Figure 1.

Construction and characteristics of Ad/TRAIL-E1. (a), Diagrams of the shuttle plasmid vector pAd/hTC-TRAIL-E1 and the oncolytic adenovector Ad/TRAIL-E1. CMV-E, CMV enhancer; BGH pA, bovine growth hormone polyadenylation signal. (b), RT-PCR analysis of TRAIL mRNA in the H1299 NSCLC cell line after treatment with PBS or adenovectors at MOIs of 1,000 vp/cell for 12 h and 24 h. Glyceraldehyde-3-phosphate dehydrogenase was used as a control. Direct PCR analysis from the same mRNA samples was shown in the middle panel. PCR products were analyzed by using 1.2% agarose gels electrophoresis. (c), E1A expression of different cancer cell lines infected with Ad/CMV-GFP, Ad/GFP-E1, Ad/TRAIL-RGD, or Ad/TRAIL-E1 at MOIs of 100 or 1,000 for 48 h. E1A was detected only in cells infected with Ad/GFP-E1 and Ad/TRAIL-E1. (d) FACS analysis for TRAIL-positive cells. H1299 cells were treated with PBS or 500 MOI of Ad/CMV-LacZ, Ad/TRAIL-RGD or Ad/TRAIL-E1. The TRAIL-positive cells were determined at 48 h after treatment. The data represent mean + SD of three assays. * ($p < 0.05$ when compared with other groups).

Figure 2.

Cell-killing effects of different vectors. (a), Crystal violet staining of H1299 cells treated for 4 days with PBS or with different doses (MOIs, left) of the adenovectors. (b) Cell viability assay. Three NSCLC cell lines, 2 colon cancer cell lines, and NHFBs were treated with various vectors for 96 h at various MOIs. Cell viability was determined by using the SRB assay. Cells treated with PBS were used as mock control (viability was set at 100%, per text). The data are

representative of four quadruplicate assays with similar results. The values represent the means $\pm$ SD.

Figure 3.

Apoptosis induction. The indicated cancer cell lines and NHFBs were treated with the different vectors at MOIs of 1,000 for 48 h. (a), The ratio of apoptotic cells was demonstrated by the percentage of cells at sub-G₁ phases which was determined by fluorescence-activated cell sorter analysis. No significant difference in the sub-G₁ percentage of NHFB cells was found ($P > 0.05$), whereas cancer cells showed a significant increase in the sub-G₁ percentage after treatment with Ad/GFP-E1, Ad/TRAIL-RGD and especially Ad/TR-E1 ($P < 0.05$). The data represent three experiments with similar results. The values represent the means $\pm$ s.d. (b), Western blot analysis of caspase-3 and caspase-8 in H460 cells 48 h after treatment with PBS (lane 1), Ad/CMV-GFP (lane 2), Ad/GFP-E1 (lane 3), Ad/TRAIL-RGD (lane 4) and Ad/TRAIL-E1 (lane 5). β -Actin was used as the loading control. The data shown are representative of three experiments with similar results.

Figure 4.

In vitro replication of oncolytic virus Ad/GFP-E1 and Ad/TRAIL-E1. (a), The viral titers in DLD1 and DLD1/TRAIL-R cells after treatment with the oncolytic virus at MOIs of 100 and 1,000 for 48 h or 96 h. (b), The viral titer in H1299 and NHFB cells after treatment with vectors at MOI of 500. The viral titer in cells treated with Ad/CMV-GFP and Ad/TRAIL-RGD at the same doses was not detectable.

Figure 5.

In vivo antitumor effect in H1299 subcutaneous xenograft tumor model. Intratumoral injection of 2.5×10^{10} viral particle/treatment was started when tumors reached 4–5 mm in diameter. The arrows indicate when treatments were given. (a), Tumor volume was monitored over days after the inoculation of tumor cells. Values represent the means $\pm$ standard error. (b), Kaplan-Meier survival curve.