Suppressing Orthotopic Pancreatic Tumor Growth with a Fiber-Modified Adenovector Expressing the TRAIL Gene from the Human Telomerase Reverse Transcriptase Promoter

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ABSTRACT

An adenoviral vector with RGD-modified fibers and expressing the human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene from the human telomerase reverse transcriptase (hTERT) promoter (designated Ad/TRAIL-F/RGD) was constructed, and its antitumor activity was evaluated in vitro and in vivo. An in vitro study showed that treatment with Ad/TRAIL-F/RGD elicited a high rate of apoptosis in human pancreatic and colon cancer cell lines that were either susceptible or resistant to conventional adenovectors. In vivo study showed that direct administration of Ad/TRAIL-F/RGD to an orthotopic implantation tumor model established in the pancreatic tails of nude mice significantly suppressed tumor growth: tumors in the animals treated with Ad/TRAIL-F/RGD were approximately eight times smaller than those in animals treated with a control vector. We also evaluated hTERT promoter activity and the effect of Ad/TRAIL-F/RGD on mesenchymal stem cells. Our results showed that transgene expression from the hTERT promoter in human bone marrow mesenchymal stem cells was minimal. No adverse effect was observed in mesenchymal stem cells treated with Ad/TRAIL-F/RGD. Together, our results suggest that Ad/TRAIL-F/RGD could become a potent therapeutic agent for the management of pancreatic cancer.

INTRODUCTION

The incidence of ductal adenocarcinoma of the pancreas is <2% of new cancers in the United States, but it is the fifth leading cause of cancer-related deaths (1). Since the first published report of a pancreatic resection in 1912 (2), the pancreaticoduodenectomy is the therapy of choice with low perioperative mortality in high-volume institutions (3). However, at the time they are diagnosed, only 10–15% of these cancers are resectable (4), and the average survival time after diagnosis is 3–6 months (5), with a 5-year survival rate for all of the stages of <4% (6). Adjuvant chemotherapy and radiation therapy have not improved survival rates; immunotherapy is being studied clinically (4).

Gene therapy may be an alternative approach for management of pancreatic cancer (7, 8). We and others have shown recently that direct introduction of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) gene into cancer cells induces apoptosis and suppresses tumor growth in vitro and in vivo (9–11). Moreover, nontransfected neighboring cancer cells can be killed by a TRAIL-mediated bystander effect (9, 12). We also reported that TRAIL-related hepatocyte toxicity could be prevented using the human telomerase reverse transcriptase (hTERT) promoter, which is highly active in >85% of human cancer cells but inactive in most somatic cells (10, 13).

However, we also found that repeated application of apoptosis-inducing adenovectors can result in the selection and expansion of resistant cells. One mechanism involved in this acquired resistance is resistance to adenovector infections (14), presumably because of low expression of the initial binding receptor, the coxsackie-adeno virus receptor (CAR; Refs. 15, 16). Reduced expression of CAR also has been reported in primary tumors, suggesting overcoming resistance to adenovirus in cancer cells is critical for future success of adenovirus-mediated cancer gene therapy (17, 18). Accumulating evidence has shown that adenoviral capsid proteins can be modified to target adenovectors to CAR-independent binding molecules. For example, modifying the adenoviral vector by incorporating the integrin-binding motif RGD (Arg-Gly-Asp sequence) into the HI loop of the adenoviral fiber protein significantly increased transduction efficiency in cells resistant to conventional adenovectors (19, 20).

To generate adenovectors that are broadly applicable to cancer therapy, we constructed an adenoviral vector, Ad/ TRAIL-F/RGD, that had the RGD sequence in the HI loop of fiber and expressed the TRAIL gene from the hTERT promoter via G AL4 gene regulatory components that can augment transgene expression from the tumor-specific promoter without losing target specificity (21).

We evaluated the efficacy of Ad/TRAIL-F/RGD in vitro in various cancer cells, including three pancreatic cancer cell lines, and in vivo in an orthotopic pancreatic tumor model. Our results
showed that Ad/TRL-F/GRG could effectively suppress the growth of orthotopic pancreatic tumors, suggesting that this vector will be useful to manage pancreatic tumors. Furthermore, the transgene expression from the hTERT promoter in human bone marrow mesenchymal stem cells was minimal, and no apoptosis induction was observed in these cells after treatment with Ad/TRL-F/GRG, even with high multiplicity of infection (MOI).

MATERIALS AND METHODS

Cell Lines and Cell Culture. Cancer cell lines used in this study were human pancreatic cell lines Capan-1, AsPC-1, and MIA PaCa-2, human lung cancer cell lines H358 and H460, human hepatoma cancer cell line Hep3B, and human colon cancer cell lines LDLI and LDLI-M. These cell lines were either obtained from the American Type Culture Collection (Manassas, VA) or maintained in our laboratory. The LDLI-M cells were selected from liver metastasis tissue after a spleen injection of the parental cell line LDLI as described previously (14). The cells were maintained in either DMEM or RPMI 1640 medium. Both media were supplemented with 10% fetal bovine serum, 1% penicillin, and streptomycin (Life Technologies, Rockville, MD). Human mesenchymal stem cells were provided by Dr. Marini and cultured in α-MEM supplemented with 20% fetal bovine serum. All of the cells were cultured at 37°C in a humidified incubator containing 5% CO2.

Adenovectors. Adenovectors Ad/CMV-GFP and Ad/ gTRL-F/GRG have been described previously (10). Ad/TRL-F/GRG was constructed by cotransfecting 293 cells with a shuttle plasmid expressing a full-length human TRL promoter and a 30-kb Cla fragment from Ad/LacZ-F/GRG (22) as described previously (23). The expansion, purification, titration, and quality analyses of all of these vectors were performed at the vector core facility at The University of Texas M. D. Anderson Cancer Center as described previously (13). All of the viral preparations were found free of the E1+ adenovirus using PCR and free of endotoxin using a Limulus amebocyte lysate endotoxin detection kit (BioWhittaker, Walkersville, MD; Ref. 13). The titer used in this study was determined by the absorbency of the dissociated virus at A260 nm [A260 nm unit = 1015 viral particles (VPs)/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 1000 VPs was equivalent to an MOI of 10–30 infectious units. Unless otherwise specified, Ad/CMV-GFP was used as the vector control, and PBS was used as a mock control.

Cell Viability Assay. Cell viability was determined using a 3-bis-[2-methoxy-4-nitro-5 sulfonyl]-2H-tetrazolium-5-carboxanilide inner salt (XTT) assay (Cell Proliferation Kit II; Roche Molecular Biochemicals, Indianapolis, IN) as described previously (13). Each experiment was performed in quadruplicate and repeated at least twice.

Flow Cytometric Assay. Fluorescence-activated cell sorting (FACS) was performed to determine in vitro GFP/TRL expression and apoptosis induction. The Capan-1, AsPC-1, MIA PaCa-2, and LDLI-M cells were plated onto 100-mm plates at a density of 1 × 106 cells/plate 1 day before infection by the virus. The cells then were infected with Ad/CMV-GFP, Ad/gTRL-F, and TRAIL-F/GRG using an MOI of 2000 VPs/cell and PBS as a mock control. After incubation for 48 h, adherent and floating cells were harvested (adherent by trypsinization) and washed with PBS. The cells then were fixed with 70% ethanol overnight and stained with propidium iodide (PI; 1 ml PI, 10 μg/ml RNase, and 9 ml PBS; PI, 50 μg/ml) before analysis to quantify the apoptotic cells. This procedure was done using flow cytometry to measure the sub-G1/G1 cellular DNA content using Cell Quest software (Becton-Dickinson, San Jose, CA).

Western Blot Analysis. The cells were washed with cold PBS and subjected to lysing in Laemmli lysis buffer. Equal amounts of lysate were separated using 10% SDS-PAGE and then transferred to Hybond enhanced chemiluminescence membranes (Amersham, Piscataway, NJ). The membranes then were blocked with PBS containing 5% low-fat milk and 0.05% Tween for 1 h or overnight at 4°C, washed three times with PBS containing 0.05% Tween, and incubated with primary antibodies for at least 1 h at room temperature. After being washed again with PBS containing 0.05% Tween, the membranes were incubated with peroxidase-conjugated secondary antibodies and developed using a chemiluminescence detection kit (ECL kit; Amersham). Rabbit antihuman caspase-8 and TRAIL were obtained from BD Biosciences PharMingen (San Diego, CA). β-Actin was used as the loading control.

Animal Study. 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 pancreatic Capan-1 cancer cells were established in 4–6-week-old nude nude mice (Charles River Laboratories Inc., Wilmington, MA) by s.c. inoculation of 4 × 106 cells into the dorsal flanks of three mice. Only single-cell suspensions with >90% viability were used for the injections. After 2 weeks, the tumors were resected aseptically and washed with PBS. Necrotic tumor tissue was cut away, and the remaining tumor was minced with scalpels into ~1.0 × 1.0 × 1.0-mm pieces. The mice were anesthetized by isoflurane inhalation and were positioned laterally. Each abdomen was sterilized with 70% ethanol; a small left abdominal flank incision was made; and the pancreas tail with the spleen was carefully exposed. The tumor piece was transplanted into the pancreas tail with an absorbable surgical polidioxanone suture 6–0 (Ethicon, Somerville, NJ). Finally, the pancreas was carefully returned to the peritoneal cavity, and the peritoneum and abdominal wall were closed in one layer with wound clips.

Two weeks after the orthotopic implantation, the mice were assigned randomly to three groups of five animals each (group 1, PBS; group 2, Ad/CMV-GFP; and group 3, Ad/TRL-F/GRG). The animals were intratumorally injected with PBS, Ad/CMV-GFP, or Ad/TRL-F/GRG. The treatments started on day 14 after surgical orthotopic implantation (SOI) and were repeated every 2 days for a total of three treatments. For each treatment, surgery was performed as described previously, and 5 × 1010 VPs in 50 μl PBS were...
injected carefully with a 27-gauge needle for both groups with adenoviral treatment. Fifty μl of PBS were injected for the control animals (mock control). Finally, the peritoneal cavity was washed with PBS.

Five weeks after SOI, mice from each group underwent microcomputed tomography examination and then were killed. At autopsy, the tumor, pancreas, liver, spleen, lung, kidneys, and brain were resected and processed for histopathologic examination. Tumor volume was determined as volume = a × b²/2 (a, largest diameter; b, smallest diameter). Blood was collected to measure serum liver enzymes aspartate aminotransferase and alanine aminotransferase from four animals from each group before the treatments, 3 days after the last treatment, and at the end of the experiment.

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

**RESULTS**

**Construction and Characterization of Ad/TRAIL-RGD.** Ad/TRAIL-F/RGD has the same bicistronic expression cassette as Ad/gTRAIL except that wild-type TRAIL cDNA is used instead of the GFP/TRA1 fusion construct described in Ad/gTRAIL (Fig. 1A; Ref. 10). In addition, Ad/TRAIL-F/RGD contains an insertion of the CDCRGDCFC sequence in the HI loop of fiber and a deletion in the E3 region from bp 28599 to 30469. The sequences of the E3 region, the fiber region, human TRAIL cDNA, and the GALA/VP16 fusion gene in the E1 region in Ad/TRAIL-F/RGD were verified by automatic DNA sequencing using purified viral DNA as templates.

The functionality of Ad/TRAIL-F/RGD was first evaluated in the human pancreas cancer cell line Capan-1. Treatment of Capan-1 cells with Ad/TRAIL-F/RGD at an MOI of 2000 VPs resulted in massive cell death (Fig. 1B). In contrast, infecting the Capan-1 cells with Ad/CMV-GFP at the same dose did not have any noticeable effect on cell viability.

**Cell-Killing Effect of Ad/TRAIL-F/RGD in Cancer Cells in Vitro.** To further test the cell-killing effect of Ad/TRAIL-F/RGD, we treated eight human cancer cell lines (Capan-1, AsPC-1, MiaPaCa-2, DLD1, DLD1-M, Hep3B, H358, and H460) with Ad/CMV-GFP, Ad/gTRAIL, and Ad/TRAIL-F/RGD at MOIs of 500 and 3000 VPs. Cells treated with PBS and Ad/GFP were used as mock and vector controls, respectively. Cell viability then was monitored over time with an XTT assay. The result showed that cell-killing effects of Ad/TRAIL-F/RGD were dramatically better than those of Ad/gTRAIL in H358, DLD1-M, H460, and MiaPaCa-2 cancer cells, all of which are relatively resistant to adenovirus infection (24), when the cells were treated at a low MOI (500 VPs; Fig. 2). In Capan-1, DLD1, Hep3B, and Cpan-1 cells, all of which are sensitive to adenovirus infection, the cell-killing effects of
Ad/gTRAIL and Ad/TRAIL-F/RGD were comparable. These results suggest that Ad/TRAIL-F/RGD is more effective than Ad/gTRAIL against cancers that are relatively resistant to adenoviruses. At a high dose (MOI, 3000), however, all of the cell lines tested are susceptible to Ad/gTRAIL and Ad/TRAIL-F/RGD, and the difference between Ad/gTRAIL and Ad/TRAIL-F/RGD was minimal (data not shown). This result also is consistent with our previous observation that at a relatively high dose, cancer cells highly resistant to adenovectors were susceptible to Ad/gTRAIL (14) because nontransduced cells can be effectively killed by bystander effect of the TRAIL gene (9, 12).

Apoptosis Induction by Ad/TRAIL-F/RGD in Cancer Cells. To determine whether Ad/TRAIL-F/RGD caused apoptosis induction, we quantified the sub-G1 population in our tested cancer cells after treatment by FACS analysis (Fig. 3A). Treatment with Ad/TRAIL-F/RGD dramatically increased the percentage of apoptotic cells. In the MIA PaCa-2 cells, which have a low level of CAR expression, the percentage of apoptotic cells was twice that of the cells treated with Ad/gTRAIL (24). The cells treated with PBS or Ad/CMV-GFP had only background levels of apoptosis (0.3–5.3%). Nevertheless, for assessment of cell-killing effect, cell viability determined by XTT assay is usually more sensitive than percentage of sub-G1 cells determined by FACS because cells that died at earlier time points may have broken into pieces that were not detected by FACS analysis but subtracted from XTT assay. To further test apoptosis induction by Ad/TRAIL-F/RGD, we transfected DLD1 cells at MOIs of 1000 and 2000 VPs/cell and harvested the cells after 24 h. Western blot analysis revealed a detectable band of TRAIL protein and a cleavage of caspase-8, the initial caspase activated at the death receptor pathway, in the Ad/TRAIL-F/RGD-treated cells only (Fig. 3; Refs. 25, 26). In contrast, no detectable TRAIL protein and cleavage of caspase-8 were observed in cells treated with PBS or Ad/CMV-GFP at the same doses.

Suppression of Tumor Growth in Vivo. To further evaluate the antitumor activity of Ad/TRAIL-F/RGD in pancreatic cancer, we established an orthotopic tumor model in 6-week-old nude nude mice (Fig. 4A). The administration of PBS, Ad/CMV-GFP, and Ad/TRAIL-F/RGD was initiated 2 weeks after the SOI of the pancreas tumor into the pancreas tail. At that time, the tumor had reached an approximate diameter of 0.5 cm and was growing invasively into the pancreas (Fig. 4A).
Animals received a dose of $5 \times 10^{10}$ VPs/tumor/treatment at days 14, 16, and 18 for a total of three injections. Computed tomography scans were performed to obtain images of the tumor in the pancreas of four animals treated with Ad/CMV-GFP and of four animals treated with Ad/TRAIL-F/RGD at 32 days after SOI. An obvious tumor mass in the abdominal cavity could be easily identified in the animals treated with Ad/CMV-GFP but not in those treated with Ad/TRAIL-F/RGD (Fig. 4B). The animals were killed 33 days after the tumor implantation, and tumor volumes were measured. Compared with the Ad/CMV-GFP treatment, treatment with Ad/TRAIL-F/RGD significantly suppressed tumor growth ($P < 0.05$). The mean tumor volume in the animals treated with Ad/TRAIL-F/RGD was eight times less than that in the animals treated with Ad/CMV-GFP. In comparison, no significant difference was found in the tumor growth between treatments with PBS and Ad/CMV-GFP. None of the mice had developed liver metastasis or peritoneal carcinomatosis at that time point.

**Minimal Toxicity of Ad/TRAIL-F/RGD in Vitro and in Vivo.** To determine possible liver toxicity after the intratumoral administration of Ad/TRAIL-F/RGD, we evaluated the liver enzymes aspartate aminotransferase and alanine aminotransferase before treatment, 3 days after the last treatment, and 1 day before killing the animals. The results of the serum liver enzyme assays of the animals treated with PBS, Ad/CMV-GFP, and Ad/TRAIL-F/RGD were within normal ranges at all of the times tested (data not shown), suggesting that the treatment was well tolerated.

We also evaluated the effects of Ad/TRAIL-F/RGD and hTERT promoter activity in human bone marrow mesenchymal stem cells. The cells were treated with PBS, Ad/CMV-GFP, Ad/gTRAIL, and Ad/TRAIL-F/RGD at MOIs of 500-5000 VPs/cell. No obvious morphologic changes were observed up to 4 days after all of the treatments, suggesting that even at an MOI of 5000 VPs/cell, Ad/gTRAIL and Ad/TRAIL-F/RGD are not toxic to mesenchymal stem cells (Fig. 5). In comparison, obvious apoptotic morphologic changes were observed in the pancreatic Capan-1 cancer cells after treatment with Ad/gTRAIL or Ad/TRAIL-F/RGD but not with Ad/CMV-GFP at an MOI of 500 VPs/cell. The mesenchymal stem cells also were observed under a fluorescent microscope for expression of the GFP or GFP/TRAIL fusion protein. GFP expression was detectable in

![Fig. 5](image-url)
Nevertheless, at a relatively high MOI, the improvement by transduction efficiencies in cells refractory to conventional adenovectors. Because low CAR expression and toxicity in primary human hepatocytes (10). In this study we constructed and evaluated the effect of an adenoviral vector that expressed the wild-type TRAIL gene from the hTERT promoter and contained an RGD sequence in the HI loop of its fiber protein (Ad/TRAIL-F/-RGD). By incorporating various useful features into a single vector, this vector might have a broader application than the vectors we reported previously. The in vitro experiments of our study showed that Ad/ TRAIL-F/-RGD is more effective than Ad/gTRAIL in killing cancer cells relatively resistant to conventional adenovectors. The in vivo experiments showed that intratumoral injection of Ad/TRAIL-F/-RGD effectively suppressed the growth of orthotopic pancreatic tumors with minimal toxicity, suggesting that this vector will be useful to manage unresectable pancreatic cancers. Moreover, our results showed that hTERT promoter activity is low in mesenchymal stem cells and that Ad/TRAIL-F/-RGD is not toxic to these cells even at high doses.

A substantial number of cancer cells are refractory to infection by adenovectors, partly because of low expression of the CAR initial binding receptor (15, 16). Nevertheless, cell resistance to this adenoviral transduction can be overcome using alternative vectors or adenoviral vectors with modified fibers or capsid proteins. For example, adenoviral vectors containing polylysine (28) or an RGD (19, 20) sequence have enhanced transduction efficiencies in cells refractory to conventional adenoviral vectors. Our results with the H358, MIA PaCa-2, and DLD1-M cells that are refractory to adenovirus infection support previous findings that the incorporation of RGD into adenoviral fiber improves transduction. Because low CAR expression also is observed frequently in tumor tissues, fiber-modified vector may have a broader application than a conventional adenovector in targeted cancer gene therapy (17, 18). Nevertheless, at a relatively high MOI, the improvement by fiber modification is not obvious (data not shown). One possible explanation is that bystander effect by the TRAIL gene can effectively compensate low transduction efficiency at the higher dosages (9, 12). Moreover, cell-killing assay is not as sensitive as reporter gene assay whose units may be amplified by enzyme-substrate interaction. For example, the levels of change cannot be more than twofold if cell killing in the Ad/gTRAIL group was already ~50%.

It will be interesting to test whether the difference between Ad/gTRAIL and Ad/TRAIL-F/-RGD observed in vitro may also be observed in vivo. This in vivo comparison is not included in the current orthotopic pancreatic tumor model because poor vector spreading and penetration inside tumors limit the effect of intratumoral injection of adenovectors. The majority of adenovectors remain in a small tumor area around the needle track after intratumoral injection. Therefore, micrometastasis models and system vector administration may be more appropriate for in vivo comparison of fiber-modified and unmodified vectors.

One concern in using the hTERT promoter for targeted cancer gene therapy is the possible leakage of promoter activity in stem cells. We found previously that hTERT promoter activity in CD34+ human bone marrow progenitor cells is low and comparable with that in other normal cells (29). In this study, we found that hTERT promoter activity also was low in human bone marrow mesenchymal stem cells. Together, our results provide additional evidence that the hTERT promoter is useful for targeted cancer gene therapy.

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