Gene Therapy in Colon Cancer Cells with a Fiber-modified Adenovector Expressing the TRAIL Gene Driven by the hTERT Promoter

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Abstract. Background: Repeated administration of adenoviral vectors can lead to cell resistance, probably because of the initial coxsackie-adenovirus receptor (CAR). Modified adenoviral vectors containing an Arg-Gly-Asp (RGD) sequence can overcome resistance. We constructed an adenoviral vector with RGD-modified fibers, expressing the TRAIL gene from the human telomerase reverse transcriptase (hTERT) promoter (designated Ad/TRAIL-F/RGD), and tested its antitumor activity in 5 colon carcinoma cell lines. Materials and Methods: Colon cancer cells were infected with Ad/CMV-GFP (vector control), Ad/gTRAIL (positive control) and Ad/TRAIL-F/RGD. PBS was used as a control. Cell viability was determined by proliferation assay. Cell-cycle analysis and quantification of Caspase-8 and TRAIL were used to identify apoptosis. Results: Treatment with Ad/TRAIL-F/RGD resulted in significantly less cell viability, increased Caspase-8 and TRAIL activity, and a greater apoptotic fraction than treatment with PBS or Ad/CMV-GFP. Conclusion: The adenoviral vector Ad/TRAIL-F/RGD could become a potent therapeutic agent for the treatment of colon carcinoma.

Cancer of the colon and rectum remains the second leading cause of cancer-related death in Europe and the USA (1). The prognosis after surgery depends on the stage grouping (UICC). The 5-year survival is only 30% to 40% in patients with a TNM stage of any T and more than 3 positive lymph nodes (2). Furthermore, the 5-year survival decreases below 10% in patients with metastases. Adjuvant chemotherapy is the gold standard for lymph node-positive patients, showing some, but not satisfactory effects on patient survival (2).

New treatment strategies are required, and gene therapy may be a new approach to cancer therapy. One focus in gene therapy is the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). We and others have recently shown that direct transfer of the TRAIL gene into human cancer cells induces apoptosis in vitro and suppresses tumor growth significantly in vivo (3-5). We also demonstrated that TRAIL-related hepatocyte toxicity can be prevented using the tumor-specific human telomerase reverse transcriptase (hTERT) promoter, which is active in more than 85% of human cancer cells, but inactive in most somatic cells (4,6). However, repeated application of apoptosis-inducing adenovectors can result in cell resistance to adenovector infections (7), presumably because of low expression of the initial binding receptor, the coxsackie-adenovirus receptor (CAR) or integrins, such as avb3, avb5, or avb1 (8,9). One technique to overcome resistance is to modify the adenoviral vector by incorporating the integrin-binding motif RGD (Arg-Gly-Asp) into the HI loop of the adenoviral fiber protein. Several reports showed significantly increased transduction efficiency in a variety of tumors (10-14).

Therefore, we constructed an adenovector that is broadly applicable to cancer therapy, designated as 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 GAL4 gene regulatory components that can augment transgene expression from the tumor-specific promoter without losing target specificity (15). In this study we evaluated the efficacy of Ad/TRAIL-F/RGD in comparison to our previously constructed adenoviral vector Ad/gTRAIL in five colon cancer cell lines.
Materials and Methods

Adenovectors. The adenoviral vectors Ad/CMV-GFP and Ad/gTRAIL have been described previously (4). Ad/TRAIL-F/RGD was constructed by co-transfecting 293 cells with a shuttle plasmid expressing a full-length human TRAIL coding sequencing from the hTERT promoter and a 30 kb ClaI fragment from Ad/LacZ-F/RGD as described previously (16). The expansion, purification, titration and quality analyses of all these vectors were performed at the vector core facility at The University of Texas M. D. Anderson Cancer Center as previously described (6). All viral preparations were found to be free of the E1+ adenovirus using polymerase chain reaction (PCR) and to be free of endotoxin using a Limulus amebocyte lysate endotoxin detection kit (BioWhittaker, Walkersville, MD, USA) (6). The titer used in this study was determined by the absorbency of the dissociated virus at A260 nm (one A260 nm unit = 10^{12} viral particles [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 multiplicity of infection (MOI) of 1000 VP was equivalent to an MOI of 10-30 infectious units. Unless otherwise specified, Ad/CMV-GFP was used as the vector control and phosphate-buffered saline (PBS) as a mock control.

Cell lines and regents. Human colon carcinoma cell lines DLD1, LOVO, SW 620, CRM and L 174T, were maintained in RPMI 1640 supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 1% glutamine and 1% penicillin and streptomycin (Gibco-BRL, Life Technologies, Inc., Grand Island, NY, USA). CRM is a liver metastatic cell line of DLD1 after inoculation into the spleen. All cells were cultured at 37°C in a humidified incubator containing 5% CO2.

Cell viability assay. Cell viability was determined using a 3-bis-(2-methoxy-4-nitro-5-sulfenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) assay (Cell Proliferation Kit II, Roche Molecular Biochemicals, Indianapolis, IN, USA), as described previously (6). Briefly, 1 x 10^4 cells/plate were seeded onto a 96-well plate, and after 24 h Ad/CMV-GFP, Ad/gTRAIL, and Ad/TRAIL-F/RGD were added with a multiplicity of infection (MOI) of 1000 VP/cell. PBS was used as a control. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO2. An XTT assay was performed daily for 5 days after the treatment. Each experiment was performed in quadruplicate and repeated at least twice. Results of representative experiments are shown as the mean of quadruplicate wells ± SD.

Flow cytometric assay. Fluorescence-activated cell sorting (FACS) was performed to determine in vitro apoptosis induction. All cell lines were plated onto 100-mm plates at a density of 1 x 10^6 cells/plate one day before treatment. The cells were then infected with Ad/CMV-GFP, Ad/gTRAIL, or Ad/TRAIL-F/RGD using an MOI of 2000 VP/cell. PBS was used as a control. After incubation for 48 h, both adherent and floating cells were harvested and washed with PBS. Cells for GFP expression were stored in media or PBS and analyzed within 4 hours. Cells for quantifying apoptosis were fixed with 70% ethanol overnight and stained with propidium iodide 1h (1 ml of PI, 10 μl of RNase, 9 ml of PBS, PI: 50 μg/ml) before analysis. This procedure was done using flow cytometry, measuring the sub-G0/G1 cellular DNA content using Cell Quest software (Becton-Dickinson, San Jose, CA, USA).

Biochemical analysis. The DLD1 cells were washed with cold PBS and subjected to lysing in Laemmli's lysis buffer. Equal amounts of lysate were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to Hybond enhanced chemiluminescence membranes (Amersham Corp., Arlington Heights, IL, USA). The membranes were then 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 Bioscience, England). Rabbit anti-human Caspase-8 and TRAIL were obtained from BD Biosciences Pharmingen (San Diego, CA, USA). β-actin was used as a loading control.

Statistical analysis. Statistical differences among the treatment groups were assessed by ANOVA using the Statistica software program (StatSoft, Tulsa, OK, USA). A value p<0.05 was considered significant.

Results

Construction and characterization of Ad/TRAIL-F/RGD. Ad/TRAIL-F/RGD has the same bicistronic expression cassette as Ad/gTRAIL (4) except that wild-type TRAIL cDNA is used instead of the GFP/TRAIL fusion construct. 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.

Cell-killing effect of Ad/TRAIL-F/RGD in colon carcinoma cells. We tested the cell-killing effects of Ad/TRAIL-F/RGD in five colon carcinoma cell lines. All cancer cell lines were sensitive to Ad/TRAIL-F/RGD and Ad/gTRAIL, and led to a significant (p<0.05) loss of viability as compared to the control groups PBS or Ad/CMV-GFP, which became evident as early as 1 day after transfection. Except for SW 620, all tumor cells treated with Ad/gTRAIL or Ad/TRAIL-F/RGD were killed within five days. The viability reducing effect was not significant between Ad/gTRAIL and Ad/TRAIL-F/RGD (Figure 1).

GFP expression and induction of apoptosis in colon carcinoma cells. We transfected all cancer cell lines with Ad/CMV-GFP and Ad/gTRAIL for evaluation of the transfection efficiency. Cells were transfected with an MOI of 2000 VP/cell and the GFP expression was measured after 48-h treatment by FACS analysis. All cell lines showed a strong transduction for Ad/CMV-GFP between
84% (L 174T) and 99% (DLD1) and for Ad/gTRAIL between 38% (DLD1) and 80% (SW 620) (Figure 2). To further evaluate the Ad/TRAIL-F/RGD caused apoptosis, we quantified the sub-G1 population in all cancer cell lines by FACS analysis. The MOI for the adenoviral treatment was set at 2000 VP/cell. Ad/gTRAIL and Ad/TRAIL-F/RGD dramatically increased the percentage of apoptotic cells in comparison to cells treated with PBS, or Ad/CMV-GFP. The apoptotic effect between Ad/gTRAIL and Ad/TRAIL-F/RGD was almost equal and cells treated with PBS or Ad/CMV-GFP had only background levels of apoptosis (1-3%) (Figure 2).

**Induction of apoptosis quantified by Caspase-8 and TRAIL expression.** To further evaluate apoptosis induction by Ad/TRAIL-F/RGD, we transfected DLD1 cells at MOIs of 1000 and 2000 VP/cell and harvested the cells after 24 h. Western blot analysis revealed a detectable band of TRAIL protein and a cleavage of the Caspase-8, the initial caspase activated at the death receptor pathway in the Ad/TRAIL-F/RGD-treated cells only at 2000 MOI (Figure 3). 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.

**Discussion**

Colorectal cancer is the second cause of cancer-related mortality in western countries, and for advanced, non-resectable tumors, treatment options are still unsatisfactory. New treatments are required and gene therapy may be an alternative approach. Modern gene therapy strategies include gene correction, immunomodulation, or virus-directed enzyme-prodrug therapy (17).

One focus in gene therapy is the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which is a death-inducing ligand belonging to the TNF cytokine superfamily including TNF-α, FasL/CD95/Apo1L, TRAIL/Apo2L and TWEAK/DR3L/Apo3L (18). These ligands are type-II transmembrane proteins, and membrane-bound TRAIL and recombinant soluble TRAIL can rapidly induce apoptosis in various cancer cells when they interact with DR4/TRAIL-R1 or DR5/TRAIL-2 death receptors (18).

We and others have recently reported that expression of the TRAIL or TRAIL/GFP fusion gene induce apoptosis and non-transfected neighboring cancer cells can be killed by a TRAIL-mediated bystander effect in a wide variety of cancer cells (4,18-20). However, one concern in gene therapy is the systemic toxicity, therefore we used a tumor-specific promoter.
with targeted gene expression. The gene of the human telomerase reverse transcriptase (hTERT) promoter is active in 85% of human cancer cells, but inactive in most somatic cells. (4). However, a limitation of tissue- or cell-type-specific promoters to target transgene expression is their weak transcriptional activity (15). A solution for this problem is the GAL4 gene regulatory system, wherein a weak, tissue-specific promoter drives expression of the GAL4/VP16 fusion protein (GV16), which activates a minimal synthetic promoter, GAL4/TATA (GT), which upstreams the TRAIL gene (15).

We recently constructed an adenoviral vector, which combines these qualities (designated as Ad/gTRAIL) and showed strong apoptosis in a variety of cancer cell lines (3,4).

However, repeated administration of adenoviral vectors, as a gene transport delivery device, can result in cell resistance to adenovirus binding mechanism, especially the initial coxsackie-adenovirus receptor (CAR) (7, 8, 21). In addition, reduced expression of CAR has also been reported in primary tumors (22). Therefore, modified vectors containing an RGD (23, 24) sequence may have a broader application than conventional adenovectors in targeted cancer gene therapy. These vectors could overcome resistance to adenoviral treatment and increase the transfection efficiency in cells with a low CAR expression.

In this study we constructed an adenoviral vector expressing the wild-type TRAIL gene from the hTERT promoter and containing an RGD sequence in the HI loop of its fiber protein (Ad/TRAIL-F/RGD) for a broader application than the vectors we previously reported.

The in vitro experiments of our study showed that Ad/TRAIL-F/RGD significantly reduces the cell viability in a variety of colon and one colon metastatic cancer cell lines, in comparison to Ad/CMV-GFP and PBS-treated cells. FACS analysis and activation of Caspase-8 and TRAIL after Ad/TRAIL-F/RGD treatment concerned these results. These experiments showed no significant difference between Ad/gTRAIL and the new vector Ad/TRAIL-F/RGD in all tested cell lines, but the advantage of the new vector depends on cells with a low CAR expression.
Our results showed that Ad/TRAIL-F/RSV leads to strong apoptosis in multiple human colon cancer cell lines and might be a new potent therapy in clinical trials for advanced cancer, because of its low systemic toxicity to hepatocytes, tumor-targeted specificity and the TRAIL-mediated bystander effect.

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References


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