Radiotherapy Sensitization by Tumor-Specific TRAIL Gene Targeting Improves Survival of Mice Bearing Human Non–Small Cell Lung Cancer

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Abstract

Purpose: To sensitize non–small cell lung cancer (NSCLC) to radiotherapy by tumor-specific delivery of tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) gene.

Experimental Design: The TRAIL was delivered to human NSCLC cell lines and normal human bronchial epithelial cells by the replication-defective adenoviral vector Ad/TRAIL-F/RGD using a tumor-specific human telomerase reverse transcriptase promoter. Cancer growth was studied using 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt and clonogenic assays. Activation of the apoptosis pathway was analyzed in a Western blot and sub-G1 DNA accumulation. A xenograft mouse lung cancer model was treated by intratumoral injections of Ad/TRAIL-F/RGD and local radiotherapy; the other groups received one of these treatments alone or a control agent. Apoptosis and TRAIL expression in tumors were also analyzed.

Results: Ad/TRAIL-F/RGD specifically targets human NSCLC cells without significant effect in normal human bronchial epithelial cells. The combination of Ad/TRAIL-F/RGD and radiotherapy significantly improved cell-killing effect in all NSCLC cell lines tested (P < 0.05). Expression of TRAIL showed a dose-dependent relationship with Ad/TRAIL-F/RGD, and radiation seemed to increase TRAIL expression. Activation of the apoptosis by TRAIL and radiation was shown by activation of caspase-9, caspase-8, caspase-3, and poly(ADP-ribose) polymerase and increased DNA sub-G1 accumulation. The combination of TRAIL and radiotherapy significantly increased apoptosis in vivo, inhibited tumor growth, and prolonged mean survival in mice bearing human NSCLC to 43.7 days compared with 23.7 days (TRAIL only) and 16.5 days (radiotherapy only; P < 0.05).

Conclusions: The combination of Ad/TRAIL-F/RGD and radiotherapy significantly improved therapeutic efficacy in suppressing NSCLC tumor growth and prolonging survival. Ad/TRAIL-F/RGD may improve the therapeutic ratio of radiotherapy in NSCLC.

Lung cancer remains the leading cause of cancer death in both men and women in the United States, with non–small cell lung cancer (NSCLC) accounting for 80% of all cases (1). Approximately 50% of patients with NSCLC have locally advanced (stage III) disease at presentation and require multimodality management. However, the median survival times for patients receiving such therapy is only 15 to 17 months (2). Radiation resistance is one of main reasons for treatment failure. Although radiation dose escalation may improve local control, considerable toxicities are associated with higher-dose radiotherapy, particularly with concurrent chemotherapy. Given the dismal outcomes of the current treatment and the considerable treatment-related toxicities in lung cancer, a novel approach with a different toxicity profile is urgently needed to sensitize lung cancer to radiotherapy.

Advances in our understanding of the molecular and cellular biology of cancer offer a broad range of possible radiosensitizing approaches. Gene therapy, for example, may be an alternative approach for treating patients with radiation-resistant lung cancer (3–5). One agent that might be very useful for such gene therapy is the tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) gene, which is able to specifically kill malignant cells but mostly spare normal cells (6–8). We and others have shown that adenoviral vector–mediated introduction of the 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 (10, 12). We also reported that TRAIL-related hepatocyte toxicity could be prevented by using the human telomerase...
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Preliminary data obtained in a breast cancer model showed that the combination of TRAIL and radiotherapy has a synergistic effect on an established human breast cancer xenograft model (14). In another study, most NSCLC tissue samples and all NSCLC cells tested were positive for telomerase expression (15). It is also known that hTERT dosage correlates with telomerase activity in human lung cancer cells (16). In addition, death receptor 4 (DR4), death receptor 5 (DR5), and TRAIL with telomerase activity in human lung cancer cells (16). In all NSCLC cells tested were positive for telomerase that the combination of TRAIL and radiotherapy has a combination.

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Laboratories (Wilmington, MA). Before tumor cell inoculation, mice (4-6 weeks of age) were purchased from Charles River. Animal experiments were done in accordance with the NIH requirements.

Cell clonogenic survival. Cells were cultured overnight in 10 cm dishes in normal culture medium and then treated with Ad/TRAIR/F-RGD or Ad/CMV-GFP at various MOIs (H1299 cells, 300 VPs; H358 cells, 100 VPs; A549 cells, 3,000 VPs; and NHBHE cells, 1,000 VPs). Cells treated with PBS alone were used as a control. After a 24-hour exposure to Ad/TRAIR/F-RGD or Ad/CMV-GFP, cells in the 10 cm dishes were irradiated with various doses of radiation in a 137Cs unit at room temperature. The irradiated cells were then seeded in triplicate onto 10 cm dishes at a density of 1,000 cells per dish to yield 50 to 200 colonies per dish. The cells were then cultured in an incubator containing 5% CO₂ at 37°C for 14 days. Individual colonies (>50 cells per colony) were fixed and stained with a solution containing 0.25% crystal violet and 10% ethanol for 10 minutes. The colonies were counted with an imaging system (FluorChem 8800 Imaging System, Alpha Innotech, San Leandro, CA) using a visible light source. Each experiment was done in triplicate and repeated at least twice.

Flow cytometry assay. Cells (2 x 10⁶) were plated into six-well plates 1 day before treatment. These cells were then treated with Ad/TRAIR/F-RGD, Ad/g-TRAIL, or Ad/CMV-GFP at various MOIs. Cells treated with PBS alone were used as a control. After a 24-hour exposure to Ad/TRAIR/F-RGD or Ad/CMV-GFP, the cells were irradiated or not treated with 5 Gy radiation in a 137Cs unit at room temperature. Three days after treatment, floating and attached cells were harvested and washed with PBS. One part was used for the analysis of GFP expression, which involved determining the percentage of GFP-positive cells using a fluorescence-activated cell sorting system (Becton Dickinson, Mansfield, MA). The second part of the sample, which was fixed by 70% ethanol overnight and stained with propidium iodide before analysis, was used to quantify the apoptotic cells. This was done using flow cytometry, which measured the sub-Go/G₀ cellular DNA content using Cell Quest software (Becton Dickinson, San Jose, CA).

Western blot analysis. Cells were cultured overnight in 10 cm dishes in normal culture medium and then treated with Ad/TRAIR/F-RGD, Ad/g-TRAIL, or Ad/CMV-GFP at a MOI of 300 VPs. After a 24-hour exposure to Ad/TRAIR/F-RGD or Ad/CMV-GFP, the cells were irradiated or not treated with 5 Gy radiation in a 137Cs unit at room temperature. Three days after the treatment, cells were washed with cold PBS and underwent lysis in Laemmli lysis buffer. Equal amounts of protein were separated by SDS-PAGE and transferred to enhanced chemiluminescence membranes (Hybond, Amersham Corp., Arlington Heights, IL). These membranes were then blocked with a buffer containing 5% fat-free milk and PBS with 0.05% Tween 20 for 1 hour or overnight at 4°C. Washed thrice with PBS with 0.05% Tween 20, and incubated with primary antibodies for at least 1 hour at room temperature. Rabbit anti-human caspase-3 and caspase-9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-human caspase-8, mouse anti-human poly(ADP-ribose) polymerase (PARP), and mouse anti-human TRAIL were obtained from BD PharMingen (San Diego, CA). After a second washing with PBS and 0.05% Tween 20, membranes were incubated with peroxidase-conjugated secondary antibodies and developed with a chemiluminescence detection kit (EnChemed Chemiluminescence kit, Amersham Bioscience, Buckinghamshire, United Kingdom). β-Actin was used as a loading control.

Animal studies. All animals in this study were maintained and animal experiments were done in accordance with the NIH requirements and institutional guidelines established for the animal care facility at The University of Texas M.D. Anderson Cancer Center. Female nude mice (4-6 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA). Before tumor cell inoculation, mice were subjected to 3.5 Gy of total body irradiation from a 137Cs radiation unit (model E-0103, U.S. Nuclear Corp.). H1299 NSCLC cells were used to establish s.c. tumors in mice. Briefly, 3 x 10⁶ cells were injected into the two hind legs of each mouse. When the average size of tumors reached 80 to 100 mm³, mice were randomly divided into six treatment groups with eight mice per group; groups were treated with PBS alone, Ad/CMV-GFP alone, Ad/TRAIR/F-RGD alone, radiation alone, Ad/CMV-GFP plus radiation, or Ad/TRAIR/F-RGD plus radiation. On day 1, each mouse was intratumorally injected with either Ad/TRAIR/F-RGD at a dose of 5 x 10¹⁰ VPs per tumor, Ad/CMV-GFP at a dose of 5 x 10¹⁰ VPs per tumor, or PBS at a volume of 0.1 mL on day 2, tumors in mice that were assigned to receive radiation were locally irradiated with a vertical 60Co–radiation beam (Theratron 780C cobalt unit, Theratronics International Ltd., Kanata, Ontario, Canada) at a dose of 5 Gy per tumor. Each mouse was given anesthesia, restrained in a custom-designed jig, and positioned in the radiation field so that only the tumor xenografts implanted on the hind legs were exposed to the radiation beam; the rest of the mouse’s body was shielded by a lead block. On day 3, each mouse that had been injected with an adenoval vector on day 1 was reimplanted with the same vector. Mice were ear-tagged so that data obtained from individual animals could be traced. Tumor dimensions were measured thrice weekly using a digital caliper. Tumor volume was calculated using the equation: V = a x b² / 2, where a is the largest diameter and b is the smallest diameter.

The mice were killed at the animal core facility in accordance with our institution’s policy when the tumor size and the mice’s general condition suggested that the mice would die within 1 week. The survival times of mice were estimated based on the date of killing.

Immunohistochemistry. Paraformaldehyde-fixed tumor sections (4 μm) were rehydrated and then incubated with a 1:200 of rabbit polyclonal antibody to TRAIL (Santa Cruz Biotechnology) for 60 minutes. The slides were stained with Envision+ System horseradish peroxidase (3,3-diaminobenzidine) by using Dakoautostainer (Dako-Cytomation, Carpeneria, CA) to detect the expression of TRAIL. Counterstaining was done with hematoxylin, and the slides were covered with Kaiser’s glycerin-gelatin. Brown color indicates TRAIL expression. Percentage of TRAIL-positive cells was counted under a light microscope (×400 magnification) in randomly chosen fields and calculated as a percentage of at least 1,000 scored cells.

Terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay. To detect apoptotic cells in tumor, we used the In situ Cell Death Detection kit, POD (Roche Applied Science, Indianapolis, IN). The staining was done according to the manufacturer’s procedures, counterstained with hematoxylin, and viewed under a light microscope. Brown color indicates apoptotic nuclei as visualized using 3,3-diaminobenzidine substrate. Apoptotic cells were counted under a light microscope (×400 magnification) in randomly chosen fields, and the apoptosis index was calculated as a percentage of at least 1,000 scored cells.

Statistical analysis. Differences among the treatment groups were analyzed by ANOVA using statistical software (Statistica, StatSoft, Tulsa, OK). A difference was considered statistically significant when P ≤ 0.05. Differences in tumor growth in vivo among the treatment groups were assessed by ANOVA with a repeated-measures module. ANOVA was done to determine statistical significance between each treatment group by using the SAS procedure mixed with SAS version 6.12 software. Survival was assessed by using the Kaplan-Meier method.

Results

Ad/TRAIR/F-RGD is more effective than Ad/gTRAIL in human non–small cell lung cancer cell lines. To test the antitumor activity of Ad/TRAIR/F-RGD, we treated H1299 cells with Ad/CMV-GFP, Ad/gTRAIL, and Ad/TRAIR/F-RGD. The cell-killing effect of Ad/TRAIR/F-RGD was analyzed by measuring cell viability via XTT assay (Fig. 1A) and by quantifying apoptotic cells via flow cytometry (Fig. 1B). Treatment of H1299 cells with Ad/TRAIR/F-RGD at 300 VPs dramatically reduced cell viability via XTT assay (Fig. 1A) and by quantifying apoptotic cells via flow cytometry (Fig. 1B).
viability compared with Ad/gTRAIL or Ad/CMV-GFP with same MOIs at days 3 to 5 ($P < 0.05$; Fig. 1A). The result was supported by flow cytometry study showing treatment of H1299 cells with Ad/TRAIL-F/RGD at MOIs of 100 to 1,000 VPs significantly increased the number of apoptotic cells compared with Ad/gTRAIL or Ad/CMV-GFP at the same MOIs ($P < 0.05$; Fig. 1B). The induction of apoptosis by Ad/TRAIL-F/RGD was confirmed by Western blot analysis (Fig. 1C). Caspase-8 and PARP cleavage were observed in cells treated with Ad/gTRAIL or Ad/TRAIL-F/RGD. However, in comparison with Ad/gTRAIL, treatment with Ad/TRAIL-F/RGD notably increased the cleavage of caspase-8 and PARP. Taken together, our data showed that Ad/TRAIL-F/RGD is more effective than Ad/g-TRAIL in cell killing and apoptosis induction in human NSCLC cells.

**Dose-dependent cell-killing effect of Ad/TRAIL-F/RGD alone in human non–small cell lung cancer cell lines but not in normal human bronchial epithelial cells.** To test the combined effect of TRAIL gene therapy and radiotherapy, we first evaluated the dose effect of Ad/TRAIL-F/RGD alone. Treatment with Ad/TRAIL-F/RGD resulted in a dose-dependent cell-killing effect at MOIs of 0 to 3,000 VPs in all cancer cell lines tested but not in NHBE cells (Fig. 2A). H358 cells were the most sensitive, H1299 cells were the next sensitive, A549 cells were the least sensitive, and NHBE cells were not sensitive. This result is consistent with the levels of transgene expression seen in the tumors treated with Ad/g-TRAIL and Ad/CMV-GFP at MOIs of 0 to 3,000 VPs (Fig. 2B). Treatment with Ad/gTRAIL or Ad/CMV-GFP resulted in similar levels of GPF-positive cells in all NSCLC cell lines but not in NHBE cells. There was a significant difference levels of GPF-positive cells between treatment with Ad/g-TRAIL versus Ad/CMV-GFP in NHBE cells ($P < 0.001$). More than 80% of NHBE cells treated with Ad/CMV-GFP at 3,000 VPs were positive for GFP, whereas only 5% of NHBE cells treated with Ad/g-TRAIL were positive for GFP (Fig. 2B), indicating tumor-specific transgene expression under the control of hTERT promoter in Ad/g-TRAIL. The level of transgene TRAIL expression in NSCLC cell was also found by Western blot analysis to depend on the vector dose (Fig. 2C).

To confirm no toxic effect in normal NHBE cells with Ad/TRAIL-F/RDG, we also evaluated apoptosis induction of Ad/TRAIL-F/RDG in NHBE cells by quantifying the sub-G1 population after treatment with Ad/TRAIL-F/RDG, Ad/g-TRAIL, and Ad/CMV-GFP using fluorescence-activated cell sorting analysis. As shown in Fig. 1D, only background levels of apoptosis were observed in NHBE cells after treatment with Ad/CMV-GFP, Ad/g-TRAIL, or Ad/TRAIL-F/RGD at various MOIs.

**Improved cell-killing effects of combined Ad/TRAIL-F/RGD and radiotherapy in non–small cell lung cancer cells but not in normal human bronchial epithelial cells.** Treatment with...
ionizing radiation (0-20 Gy) alone led to a dose-dependent inhibition of cell growth in the three NSCLC cell lines and NHBE cells according to the results of the XTT assay (Fig. 3). We then studied whether the combined treatment of ionizing radiation and Ad/TRAIL-F/RGD could inhibit profoundly the growth of these cells. Cells treated with PBS and Ad/CMV-GFP were used as mock and vector controls, respectively. Improved inhibitory effect on cell growth was observed in three NSCLC cell lines treated with various doses of Ad/TRAIL-F/RGD and ionizing radiation. In contrast, there was no difference observed among treatment groups with various doses of radiation combined with Ad/TRAIL-F/RGD, Ad/CMV-GFP, and PBS in NHBE cells, indicating no radiation sensitization in NHBE cells with Ad/TRAIL-F/RGD. Figure 3 shows representative data. In H1299 cells, a single treatment with 5 Gy of ionizing radiation alone caused 30.9% growth inhibition, and treatment with Ad/TRAIL-F/RGD alone (at a MOI of 300 VPs) caused 38.7% growth inhibition; however, the combined treatment caused 73.8% growth inhibition according to the results of the XTT assay. A similar growth inhibition was observed with the other two cancer cell lines but not in NHBE cells (Fig. 3). The data thus showed that combined Ad/TRAIL-F/RGD and radiation improved cell-killing effect on NSCLC cell lines but not in NHBE cells.

To confirm the combination effect of TRAIL gene therapy and radiotherapy, we conducted a clonogenic formation assay in the three NSCLC cell lines and NHBE cells after treatment with radiotherapy, Ad/TRAIL-F/RGD, or both. PBS and Ad/CMV-GFP were again used as mock and vector controls, respectively. Compared with the control group or the groups receiving single-agent treatment, the group receiving a combination of Ad/TRAIL-F/RGD and radiotherapy showed significantly reduced clonogenic formation (Fig. 4A and B; \( P < 0.05 \)) in the three NSCLC cell lines. Nevertheless, combination of Ad/TRAIL-F/RGD and radiotherapy resulted in almost similar colony formation compared with radiotherapy plus Ad/CMV-GFP group and radiotherapy plus PBS group in NHBE cell lines. In H1299 cells, 5 Gy of radiation inhibited 50.2% of colony formation and Ad/TRAIL-F/RGD treatment at a MOI of 300 VPs suppressed 30.1% of colony formation. However, the combination of radiation and Ad/TRAIL-F/RGD treatment inhibited 99.5% of colony formation. A similar colony formation inhibition was observed in the other two cancer cell lines (Fig. 4B). The different percentages of inhibition shown by the XTT and clonogenic assays were due to the different sensitivities of these two assays with the different treatments. These results suggested that TRAIL gene therapy significantly sensitize NSCLC cells to radiotherapy. Interestingly, radiotherapy increased TRAIL gene expression at different concentrations of the vectors (Fig. 2C).

Enhanced apoptosis induction and activation of the apoptotic signal transduction pathway by the combination of tumor necrosis factor–related apoptosis-inducing ligand gene therapy and radiotherapy. To test whether the enhanced cell killing produced by combination treatment is due to apoptosis, we quantified the sub-G₁ population of H1299 cells after treatment with TRAIL gene therapy and radiotherapy in a fluorescence-activated cell sorting analysis. As shown in
Fig. 5A, Ad/TRAIL-F/RGD alone or radiation alone induced a <5% sub-G₁ accumulation. However, combined treatment with Ad/TRAIL-F/RGD and radiation resulted in a substantially increased sub-G₁ accumulation (10.2%). These data suggested that apoptosis is significantly enhanced by the combination of TRAIL gene therapy and radiotherapy.

To further elucidate the apoptotic signal transduction pathway used by TRAIL gene therapy and radiotherapy, we did a Western blot study to evaluate the activation of caspase-8, caspase-3, and caspase-9 and the cleavage of PARP after treatment with PBS, Ad/CMV-GFP alone, Ad/TRAIL-F/RDG alone, radiotherapy alone, and radiotherapy plus Ad/CMV-GFP or Ad/TRAIL-F/RDG. H1299 cells were harvested 4 days after treatment with Ad/TRAIL-F/RGD or Ad/CMV-GFP and 3 days after radiotherapy, and the activation of caspase-8, caspase-3, and caspase-9 and the cleavage of PARP were analyzed. Treatment with Ad/TRAIL-F/RGD alone resulted in the cleavage of caspase-8, caspase-3, caspase-9, and PARP as expected (Fig. 5B). The combined treatment with Ad/TRAIL-F/RGD and radiation resulted in the cleavage of caspase-8, caspase-3, caspase-9, and PARP that was slightly increased over that produced by treatment with Ad/TRAIL-F/RGD alone (Fig. 5B).

This result indicated that apoptosis was responsible for the cell death produced by the combination of TRAIL gene therapy and radiotherapy and also suggested that the combination treatment enhanced apoptosis.

Inhibition of tumor growth in vivo and prolongation of mouse survival by combined Ad/TRAIL-F/RDG treatment and radiotherapy. We then tested the therapeutic efficacy of the combination of Ad/TRAIL-F/RDG treatment and radiotherapy in vivo in a human NSCLC xenograft mouse model. As shown in Fig. 6A, radiotherapy had a minimal inhibitory effect and Ad/TRAIL-F/RDG alone had a moderate inhibitory effect on tumor growth at the tested dose regimens; however, the combination of Ad/TRAIL-F/RDG and radiation significantly inhibited tumor growth compared with treatment with PBS alone, radiotherapy alone, Ad/TRAIL-F/RDG alone, Ad/CMV-GFP alone, or Ad/CMV-GFP plus radiotherapy (day 14; P = 0.00907).

The mouse survival data also showed a significant prolongation of survival time for the combination of Ad/TRAIL-F/RDG and radiotherapy, with a mean survival of 43.7 days compared with 23.7 days for Ad/TRAIL-F/RDG alone, 16.5 days for radiation alone, 15.4 days for Ad/CMV-GFP alone, and 15.7 days for PBS.
Fig. 4. Enhanced cell-killing effects of combined Ad/TRAIL-F/RGD and radiotherapy in NSCLC cells but not in NHBE cells by the colony-forming assay. The influence of Ad/TRAIL-F/RGD on radiosensitivity was examined based on the clonogenic survival of cell lines after exposure to PBS, Ad/CMV-GFP (GFP), Ad/TRAIL-F/RGD (TRAIL), radiation (RAD), Ad/CMV-GFP and radiation (GFP+RAD), and Ad/TRAIL-F/RGD and radiation (TRAIL+RAD). The MOIs for the cells were as follows: H1299 cells, 300 VPs; H358 cells, 100 VPs; A549 cells, 3,000 VPs; and NHBE cells, 1,000 VPs. A, representative dishes by colony-forming assay of four cell lines at 14 days after treatment. B, relative clonogenic survival of four cell lines. Each experiment was done in triplicate and repeated at least twice. Columns, mean results of triplicate assays; bars, SD.

Fig. 5. Enhanced apoptosis induction and activation of the apoptotic signal transduction pathway by the combination of TRAIL gene therapy and radiotherapy. A, fluorescence-activated cell sorting was used to quantify apoptotic cells 4 days after treatments. The percentage of sub-G1 cells appears in each panel. Data from one of two experiments yielding similar results. B, Western blot analysis of cleavage of PARP, caspase-8, caspase-9, and caspase-3 in H1299 cells 4 days after Ad/TRAIL-F/RGD treatment combined with radiotherapy. Cells were treated with PBS (lane 1), radiation (5 Gy; lane 2), Ad/CMV-GFP (MOI of 300 VPs; lane 3), Ad/CMV-GFP (MOI of 300 VPs) plus radiation (5 Gy; lane 4), Ad/TRAIL-F/RGD (MOI of 300 VPs; lane 5), and Ad/TRAIL-F/RGD (MOI of 300 VPs) plus radiation (5 Gy; lane 6). β-Actin was used as a loading control.
alone ($P = 0.00002$; Fig. 6B). Treatment with Ad/TRAIL-F/RGD or Ad/TRAIL-F/RGD plus radiotherapy significantly lengthened survival time compared with treatment with PBS only, control vector only, radiation only, or control vector plus radiation ($P = 0.00186$). Furthermore, combined treatment with Ad/TRAIL-F/RGD and radiation prolonged survival time significantly compared with treatment with Ad/TRAIL-F/RGD alone ($P = 0.03354$). Half of the mice treated with combined Ad/TRAIL-F/RGD and radiation remained alive for >48 days compared with the mice treated with Ad/TRAIL-F/RGD alone, all of which died within 30 days, and the mice treated with radiotherapy alone all of which died within 20 days. We observed no marked treatment-related toxicities affecting the weight of mice or their general behavior.

**Combination of Ad/TRAIL-F/RGD and radiation-enhanced apoptosis induction in vivo.** To assess apoptosis induction in vivo, we did terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining on tumor sections 3 days after radiation in all six treated groups (Fig. 7A). Brown color indicates apoptotic nuclei as visualized using 3,3′-diaminobenzidine substrate. Apoptotic cells were counted under a light microscope in randomly chosen fields, and the apoptosis index was calculated as a percentage of at least 1,000 scored cells. As shown in Fig. 7B, combination of Ad/TRAIL-F/RGD and radiation resulted in a significantly higher apoptotic index (29.8%) compared with Ad/TRAIL-F/RGD alone (13.5%), radiation alone (9.7%), and radiotherapy plus Ad/CMV-GFP (10.3%) after subtracting the background level of 2.2% ($P = 0.00016$). We also determined TRAIL protein expression by immunohistochemical analysis of tumor specimens from same six treated groups. We observed strong expression of TRAIL protein in tumor tissues obtained from animals that were treated with Ad/TRAIL-F/RGD combined radiation or treated with Ad/TRAIL-F/RGD alone (Fig. 7A and C). We did not observe TRAIL expression in tumors that were treated with PBS or radiation alone or GFP alone or GFP plus radiation as expected. The percentage of positive cells in tumor tissues treated with Ad/TRAIL-F/RGD plus radiation was obviously higher (30.1%) than that treated with Ad/TRAIL-F/RGD alone (16.8%; $P = 0.00306$). This result also is consistent with our results in vitro (Fig. 2C) that radiotherapy increased TRAIL gene expression.

**Discussion**

In this study, the tumor-specific TRAIL gene targeting achieved by using Ad/TRAIL-F/RGD was found to sensitize
lung cancer cells to radiotherapy, with significantly increased tumor growth inhibition and survival of mice bearing a human NSCLC xenograft. For the radiotherapy (5 Gy) only group, the tumor growth inhibition effect was minimal, and there was no survival benefit; the mean survival was 16.5 days in mice receiving radiotherapy only compared with 15.7 days in PBS-treated mice. These data suggest that the NSCLC cell line H1299 is resistant to this dose of radiotherapy. Ad/TRAIL-F/RDG alone moderately improved the tumor inhibition effect and prolonged the survival time of mice. However, combination of radiotherapy and Ad/TRAIL-F/RDG achieved a dramatic improvement with more than doubled mean survival (43.7 days).

Of further importance, there were no observable toxicities noted in the combination treatment group compared with controls based on mice weight and general behavior. The combination of Ad/TRAIL-F/RDG and radiotherapy may serve as a novel approach to increasing the therapeutic ratio of radiotherapy in NSCLC that is radiation resistant.

In terms of toxicity, radiotherapy commonly causes acute adverse effects, including esophagitis, pneumonitis, and chronic conditions, such as lung fibrosis, esophageal stricture, and fistula; these complications are the major dose-limiting toxicities of radiotherapy. Gene therapy, in contrast, causes different toxicity profiles, with common systemic reactions, such as low-grade fever (5), which do not overlap with radiotherapy-induced adverse effects. Therefore, combined gene therapy and radiotherapy may improve therapeutic efficacy on cancer cells, with tolerable toxicities.

Among the family of death receptor ligands, tumor necrosis factor and Fas have been extensively investigated, and a phase I clinical trial has been completed that assessed the effect of intratumoral injections of tumor necrosis factor delivered by an adenoviral vector (29). However, potential ischemic and hemorrhagic reactions in normal tissues and liver toxicity were of major concern (30, 31). Treatment with TRAIL is a more potent cancer therapy that causes little toxicity in normal tissues (8, 9, 32); the only reported toxicity is the apoptosis of human primary hepatocytes and human brain cells (33–35). However, more detailed studies particularly in vivo are needed to further clarify the anticancer activity of TRAIL delivered by different biochemical preparations of TRAIL proteins and the possible toxicities in humans (35, 36).

To minimize such potential toxicities, we developed a tumor-specific adenoviral vector–mediated TRAIL gene delivery system by expressing the TRAIL gene from the hTERT promoter via GAL4–GAL-regulatory components that can augment transgene expression from the tumor-specific promoter without losing target specificity (11, 25, 28). As shown in our data, TRAIL under the control of tumor-specific hTERT induced apoptosis, inhibited growth, and sensitized radiotherapy in lung cancer cells but spared normal cells, such as NIHBE cells (Figs. 1D, 2A and B, 3, and 4). The heterogeneity of human cancer cells is an additional problem because some cells do not have an adenoviral receptor in their cell membrane, thus hindering adenoviral vector delivery. To overcome this obstacle, we inserted a gene sequence containing the integrin-binding RGD motif sequence into the HI loop of the adenoviral fiber. Compared with previous vector Ad/g–TRAIL without RGD, Ad/TRAIL-F/RDG significantly improved cancer inhibition effect and apoptosis induction without losing its tumor specificity (Fig. 1).

The hTERT promoter has the ability to specifically target cancer cells. However, its promoter strength may not be strong enough to express the large amount of TRAIL required to kill cancer cells in patients with lung cancer. To increase promoter strength, a GAL4VP16 fusion protein, a very strong transcription factor for GT, was coexpressed in this construct. In this way, expression of GAL4VP16 under the control of tumor-specific hTERT promoter activates the GT promoter, which initiates TRAIL expression. Our preliminary data from the use of this new construct showed both the increased efficacy of gene delivery without a loss of tumor specificity and the minimally detectable gene expression in normal tissues, even bone marrow stem cells that are considered to have the highest hTERT promoter activity among all the normal tissues (26).

An enhanced anticancer effect of the combination of TRAIL protein therapy with radiotherapy has been seen in various other cancer cells, including breast (14), colon (37), leukemia, and lymphoma (38) cells, in several studies published over the past 4 years. For example, Chinnaiyan et al. (14) examined TRAIL protein therapy in combination with radiation therapy in a breast cancer model. An enhanced apoptotic response was tested in several human breast cancer cell lines and in an established breast cancer xenograft mouse model. The results showed an enhanced tumor growth delay in response to combination treatment compared with either treatment alone. Enhanced apoptosis was also observed in histologic sections taken from these tumors after the combination treatment. No significant toxicity was observed in the mice that received systemic treatment with TRAIL protein (14). This synergistic effect was found to be p53 dependent and may be the result of radiation-induced up-regulation of the TRAIL receptor DR5. In our study, all the three NSCLC cell lines tested no matter what are their p53 status (A549 line, which contains the wild-type p53 gene, and H1299 and H358 lines, which have an internal homozygous deletion of the p53 gene and a mutated p53 gene, respectively) showed radiation sensitization by Ad/TRAIL-F/RDG, indicating p53-independent pathway.

Ravi and Bedi (37) showed that the TRAIL protein sensitizes human colon adenocarcinoma cell lines to radiation. TRAIL sensitized HCT116 cells to radiation independently of their p53 status. However, the Bax-deficient line was insensitive to TRAIL alone, radiation alone, and their combination. Gong and Almasan (38) tested the efficacy of the combination of TRAIL protein therapy and radiation in human leukemia and lymphoma cell line treated in vitro. The combination of radiation and the recombinant TRAIL protein had a synergistic effect on the loss of clonogenic survival in both cell lines. Interestingly, data from a study of leukemic cell systems showed that the TRAIL protein sensitized malignant but not normal erythroblastic cells to radiotherapy (39).

Although TRAIL-mediated gene therapy has shown promise, certain cancer cells, especially those that make up highly malignant tumors, are TRAIL resistant. The mechanisms of the resistance to TRAIL-induced apoptosis include dysfunctions of DR4 and DR5, which are essential for TRAIL binding to cells; a defect of either the adaptor protein Fas-associated death domain or caspase-8, which are essential for the TRAIL-induced apoptotic signal transduction pathway, overexpression of Bcl-2; or a lack of Bax or Bak (40). As shown by the results of our clonogenic assay, H549 cells are resistant to Ad/TRAIL-F/RDG therapy, with a MOI of 3,000 VPs required inhibit only 33.6%
Fig. 7. Ad/TRAiL-F/RGD combined with radiotherapy enhances apoptosis in vivo. A. representative fields of TRAIL expression by immunohistochemistry (right) and tumor cell apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay (left) in H1299 tumors receiving various treatments harvested 3 days after radiation.
the cancer cells; in contrast, 63.3% inhibition of H358 cells can be achieved with Ad/TRAIL-F/RDG at a MOI of only 100 VPs. However, the combination of Ad/TRAIL-F/RDG and radiotherapy (5 Gy) inhibited 90.5% of colony formation in H549 cells. These data suggest that radiotherapy is also useful as a tool to overcome TRAIL resistance in gene therapy.

Several mechanisms explain the observed enhanced therapeutic effects between the TRAIL gene and radiotherapy. First, the TRAIL gene and radiation activate distinct apoptotic pathways, which when jointly triggered result in an amplified response (41, 42). TRAIL binds with its cell surface receptors and then mediates the recruitment of the Fas-associated death domain adapter molecule to the receptor complex. This complex directly activates caspase-8 (43) and subsequently activates caspase-3 resulting in activation of cascade of caspases (44). In addition, caspase-8 can also activate Bid that triggers cytochrome c release, with subsequent activation of caspase-9 and caspase-3, thereby strongly amplifying the initial apoptotic signal (45). In contrast to receptor-induced apoptosis, radiation directly damages DNA and triggers p53-mediated transcriptional activation of Bax, Bak, Noxa, and Puma, resulting in the mitochondrial damage by breakdown of the mitochondrial membrane potential and release of cytochrome c (46–48). Cytoplasmic cytochrome c forms a complex with Apaf-1 and dATP, leading to activation of caspase-9 (49). Activated caspase-9 subsequently activates caspase-3 and then again activates the cascade of caspases. Alternatively, radiation may directly damage cellular membrane resulting in the release of ceramide (50). Ceramide, once released into the cytoplasm, can directly damage mitochondrial membrane and then stimulate the initiation of apoptosis by releasing cytochrome c (51). This mitochondrial pathway could be p53 independent. Triggering both mitochondrial-dependent and mitochondrial-independent pathways simultaneously explain the effective therapeutic response seen in vitro and in vivo as our data showed.

Another source of the enhanced anti-tumor effects of combined radiotherapy and gene therapy may be that ionizing radiation improves the transfection/transduction efficiency and transgene integration as shown by our and other studies (52–54). Moreover, gene therapy and radiotherapy target at different phases of the cell cycle, with the S phase of the cell cycle being most responsive to gene therapy and the M and G2 phases being most radiosensitive (55). In addition, it has been reported that radiation and chemotherapy can cause up-regulation of expression of the DR4 or DR5 protein or both (14, 56, 57). By expressing more of the TRAIL receptor, cells may become more sensitive to TRAIL therapy. Further experimentation is needed to confirm this mechanism in radiotherapy.

In summary, our data showed that tumor-specific TRAIL gene targeting can sensitize NSCLC cells to radiotherapy and thus improve survival while spare the normal cells. Ad/TRAIL-F/RDG may improve the therapeutic ratio of radiotherapy, which is crucial for radiation-resistant NSCLC. In addition, radiotherapy may also overcome the TRAIL resistance in highly malignant cancer cells.

Our results provide important preclinical evidence for the future design of multimodality clinical trials that use combined TRAIL gene therapy and radiotherapy. The combination of Ad/TRAIL-F/RDG gene therapy and radiotherapy may be a feasible and effective method for the treatment of NSCLC, particularly for radiation-resistant cancers.

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