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Decoy Receptor-2 Small Interfering RNA (siRNA) Strategy Employing Three Different siRNA Constructs in Combination Defeats Adenovirus-Transferred Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand Resistance in Lung Cancer Cells

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ABSTRACT

Tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) selectively induces apoptosis in cancer cells but not in normal cells. However, studies have indicated that more than half of human tumors exhibit TRAIL resistance. Although the mechanism of TRAIL resistance is not understood, it represents a barrier to any TRAIL-mediated gene therapy approach. In addition, no correlation between TRAIL receptor (TRAIL-R) expression profile and TRAIL resistance has been demonstrated in cancer cells. In this study, three different lung cancer cell lines and three different primary cell cultures established from patients with lung cancer (two patients with squamous cell lung carcinoma and one with adenocarcinoma) were screened for sensitivity to adenoviral delivery of TRAIL. Whereas TRAIL-resistant primary lung cell cultures and the A549 lung cancer cell line exhibited high levels of surface decoy receptor-2 (DcR2/TRAIL-R4) expression, TRAIL-sensitive lung cancer cell lines (HBE and H411) failed to express it. A DcR2 short interfering RNA (siRNA) approach involving three different siRNA constructs in combination downregulated DcR2/TRAIL-R4 expression and sensitized lung cancer cells to TRAIL-induced apoptosis. Immunohistochemical staining of samples from 10 patients with lung carcinoma suggested that high-level DcR2/TRAIL-R4 expression is a common phenotype observed in patients with non-small cell lung carcinoma.

OVERVIEW SUMMARY

Lung cancer causes the highest rate of cancer-related death among both men and women. Chemotherapy and radiotherapy are inadequate in increasing patient survival as they both require p53 for their antitumor activity. Death ligands, such as tumor necrosis factor (TNF)-related apoptosis inducing ligand (TRAIL), induce apoptosis regardless of the p53 status of cancer cells. Despite this, TRAIL resistance observed in many tumors represents a handicap for any gene therapy approach targeting cancer. In this study, the A549 lung cancer cell line and primary cell cultures established from three different patients manifested high levels of surface decoy receptor-2 (DcR2/TRAIL-R4) expression and showed resistance to adenoviral delivery of TRAIL. On the other hand, a small interfering RNA (siRNA) cocktail containing three different oligonucleotides designed against DcR2/TRAIL-R4 resulted in the sensitization of lung cancer cells to adenoviral delivery of TRAIL. This study demonstrates that the modulation of TRAIL receptor profiles of cancer cells represents a new therapeutic approach to sensitize cancer cells to TRAIL.

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INTRODUCTION

LUNG CANCER is the world's leading cause of cancer deaths and causes more deaths than the next three most common cancers (colon cancer, breast cancer, and prostate cancer) combined (Jemal *et al*., 2002). Approximately 1 million people worldwide die of lung cancer yearly (Carney, 2002). On the basis of morphology, lung cancer is divided into two major histological types: non-small cell lung cancer (NSCLC) and small cell lung carcinoma (SCLC). Eighty percent of lung cancer is NSCLC, and 20% is SCLC (Jemal *et al*., 2002). Despite conventional treatment options such as surgery, chemotherapy, and radiotherapy, the 5-year survival rate among patients with NSCLC is only 14%, and the survival rate drops to 5–10% for patients with SCLC (Johnson *et al*., 1990). Because more than 50% of human tumors manifest p53 loss during tumorigenesis, tumors eventually acquire resistance to both radiotherapy and chemotherapy.

Because death ligands induce apoptosis independent of the p53 status of cancer cells, gene therapy approaches involving the administration of death ligands represent a feasible choice for the treatment of radioresistant and chemoresistant cancer cells (Herr and Debatin, 2001). All death ligands tested, including tumor necrosis factor (TNF) (Sanlioglu *et al*., 2004) and Fas ligand (FasL) (Nagata, 1997), efficiently kill cancer cells. Unfortunately, these agents have systemic toxicity, limiting their potential use for cancer gene therapy. In contrast, a novel death ligand, TNF-related apoptosis-inducing ligand (TRAIL) (Wiley *et al*., 1995), has been reported to selectively kill cancer cells without causing any harm to normal cells (Nagane *et al*., 2001). The observation that at least one of the two death receptors is expressed in the majority of locally nonresectable stage III NSCLCs further encourages the use of TRAIL as a death-inducing ligand for lung cancer (Spierings *et al*., 2003). Thus, TRAIL-mediated gene therapy represents an attractive approach for attacking cancer cells without generating systemic toxicity.

Five different receptors interact with TRAIL: TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, TRAIL-R4/DcR2, and osteoprotegerin (Abe *et al*., 2000; Sheikh and Fornace, 2000). TRAIL-R1/DR4 and TRAIL-R2/DR5 are the genuine death receptors inducing apoptosis whereas TRAIL-R3/DcR1 and TRAIL-R4/DcR2 function as decoy receptors and do not transmit death signals (Sheridan *et al*., 1997). Activation of TRAIL-R1/DR4 or TRAIL-R2/DR5 by trimeric TRAIL leads to the recruitment of Fas-associated death domain protein (FADD) to the membrane. FADD then recruits procaspase-8 to form the death-inducing signaling complex (DISC). DISC activates the caspase cascade, pushing cells into apoptosis. Caspase-8 is the first apical caspase activated by TRAIL signaling. Caspase-8 in turn activates caspase-3 directly or causes cytochrome *c* release from mitochondria through Bid cleavage. Cytochrome *c* binds to apoptotic protease-activating factor-1 (APAF1) and activates caspase-9, leading to the activation of effector caspases such as caspase-3. Finally, DNA fragmentation and cell death become inevitable.

The fact that TRAIL specifically kills malignant cells but not normal cells led to the testing of systemic TRAIL administration for cancer therapy. However, a significant fraction of the tumor cells displayed TRAIL resistance and the mechanism of TRAIL resistance is not understood (Griffith *et al*., 1998; Sanlioglu *et al*., 2003). Resistance to TRAIL-induced apoptosis can occur at different levels in the TRAIL signaling cascade. First of all, death receptor malfunction due to genetic mutations can lead to TRAIL resistance (Lee *et al*., 1999; Ozoren *et al*., 2000). In addition, death signals cannot be relayed without the proper function of FADD and caspase-8 in the death-inducing signaling complex (Kuang *et al*., 2000; Cheng *et al*., 2006; O'Flaherty *et al*., 2006). Last, the induction of Bcl-2, Bcl-X_L, and inhibitors of apoptosis (IAPs) as well as loss of Bax and Bak function may result in TRAIL resistance (Kandasamy *et al*., 2003; Zender *et al*., 2005; Hamai *et al*., 2006; Petrella *et al*., 2006). Thus, understanding the molecular mechanism of TRAIL resistance in cancer cells is essential to resolve some obstacles associated with the clinical application of TRAIL as a therapeutic agent. So far, no direct connection between the expression profile of TRAIL receptors and TRAIL resistance has been established in cancer cells. In this study, three independent lung cancer cell lines (A549, H411, and HBE) and primary lung cancer cell cultures established from three different patients were tested for sensitivity to TRAIL-induced apoptosis, using an adenoviral vector system (Ad5hTRAIL). Furthermore, TRAIL receptor compositions of lung cancer cells were revealed by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) and flow cytometric analysis. Three different small interfering RNA (siRNA) constructs were generated to knock out the relevant TRAIL decoy receptor expression to reveal its connection to TRAIL resistance in lung cancer cells. Last, an immunohistochemical analysis was performed to determine TRAIL receptor expression profiles of 10 patients with lung cancer.

MATERIALS AND METHODS

Amplification of first-generation recombinant adenoviral vectors

Amplifications of first-generation recombinant adenoviral vectors such as Ad5hTRAIL (Griffith *et al*., 2000), AdEGFP (Sanlioglu *et al*., 2004), and AdCMVLacZ (Sanlioglu and Engelhardt, 1999) were performed as described previously (Engelhardt *et al*., 1993). After CsCl banding and vector purification, adenoviral vectors were kept at −80°C in 10 m*M* Tris containing 20% glycerol. The Ad5hTRAIL construct was used to overexpress hTRAIL in lung cancer cells. Adenoviral vectors expressing the β-galactosidase gene (AdCMVLacZ) were used as a negative control. A_{260} measurements indicated that the particle titers of adenoviral stocks were in the range of 10^{13} DNA particles/ml. Functional titers were measured by plaque titering on 293 cells and by expression assays for encoded proteins. Typically, the particle:plaque-forming unit ratio was equal to 50. All three cell lines used in this work are non-small cell lung carcinoma cell lines of human origin. Specifically, A549 and H411 are adenocarcinoma cell lines. HBE (also known as 16HBE) is a simian virus 40 (SV40) large-T antigen-transformed epithelial cell line derived from human bronchial epithelium.

Efficacy of first-generation adenoviral vector transduction of lung cancer cells

Briefly, lung cancer cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), sodium bicarbonate (2.2 g/liter), 1 m*M* L-glutamine, and 1% penicillin–streptomycin–amphotericin mixture (PSA), using Thermo Scientific Steri-Cult incubators (Thermo Fisher Scientific, Waltham, MA). Adenoviral vectors expressing the enhanced green fluorescent protein (EGFP) reporter gene (AdEGFP) were transferred into lung cancer cells at increasing multiplicities of infection (MOIs) and cells were kept at 37°C in RPMI 1640 without FBS. An equal volume of RPMI 1640 supplemented with 20% FBS was added to increase the serum concentration in the medium to 10% 2 hr after infection. The percentage of $EGFP^+$ cells was determined by fluorescence microscopy and subsequently by flow cytometry 48 hr after infection. Cell viability was assessed by the propidium iodide exclusion technique.

Live/dead cell discrimination

Discrimination of live cells from dead cells was performed with a LIVE/DEAD viability/cytotoxicity kit from Invitrogen Molecular Probes (Eugene, OR). This assay is based on the use of calcein AM and ethidium homodimer-1 (EthD-1). Calcein AM is a fluorogenic substrate for intracellular calcein esterase. It is modified to a green fluorescent compound (calcein) by active esterase in live cells with intact membranes, and thus serves as a marker for viable cells. Unharmed cell membranes do not allow EthD-1, a red fluorescent nucleic acid stain, to enter inside the cell. However, cells with damaged membrane take up the dye and stain positive.

Quantitative real-time RT-PCR assays to detect human TRAIL receptor transcripts

Gene quantification was performed with an Applied Biosystems 7500 real-time PCR system running SDS software (Applied Biosystems, Foster City, CA). Total RNA from lung cancer cells was extracted with TRIzol reagent (Invitrogen Life Technologies, Gaithersburg, MD). TaqMan reverse transcription reagents (cat. no. N8080234; Applied Biosystems) were used to reverse transcribe 2 μg of total RNA. Previously described TRAIL death receptor (Karacay *et al*., 2004) and TRAIL decoy receptor (Sanlioglu *et al*., 2005, 2006) primer and probe sequences were used. Both the ribosomal RNA (rRNA) primers and probes were obtained from Applied Biosystems (cat. no. 4308329; Applied Biosystems) and served as an internal control in the same reaction. The $\Delta\Delta C_t$ method as described by Applied Biosystems was used to determine the relative quantities of TRAIL receptors for each sample. The TaqMan PCR was carried out as described by the manufacturer (cat. no. N8080228; Applied Biosystems). For the reverse transcription step, a 50-μl reaction mixture was prepared in reverse transcription buffer with 2.25 m*M* $MgCl_2$, dNTPs (50 μM each), 2.5 μM random hexamer, RNase inhibitor (0.6 U/μl), and reverse transcriptase (1.25 U/μl) diluted in RNase-free distilled water. The thermal cycling conditions were 10 min at 25°C, followed by 60 min at 48°C. TaqMan PCR was performed with TaqMan universal master mix with 50 pmol of primer and probe mixture and 250 ng of cDNA. The thermal cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. Each reaction yielded a specific threshold cycle (C_t). ΔC_t values were then calculated for each receptor by taking the difference between the C_t values of the TRAIL receptors and that of rRNA internal controls. Apart from this, a standard curve was established on the basis of serial dilutions of rRNA. The comparative $\Delta\Delta C_t$ calculation was then performed by finding the difference between the ΔC_t value of each receptor and that of 25 pg of rRNA. Final relative expression levels were estimated by the formula $2^{-\Delta\Delta C_t}$. By doing this, relative expression levels of TRAIL receptors are normalized to that of 25 pg of ribosomal RNA.

Flow cytometric detection of TRAIL receptor protein expression on the cell surface

TRAIL receptor protein expression on the cell surface was detected with an anti-TRAIL receptor flow cytometry set (cat. no. ALX-850-273-KI01; Alexis Biochemicals, Lausen, Switzerland). This kit contains 100 μg of monoclonal antibody (mAb) to TRAIL-R1 (clone HS101; cat. no. ALX-804-297A), TRAIL-R2 (clone HS201; cat. no. ALX-804-298A), TRAIL-R3 (clone HS301; cat. no. ALX-804-344A), and TRAIL-R4 (clone HS402; cat. no. ALX-804-299A). Primary antibodies were used at a concentration of 5 μg/ml. Biotinylated goat anti-mouse IgG1 (cat. no. ALX-211-202; Alexis Biochemicals) served as a secondary antibody. Streptavidin–phycoerythrin (PE) (cat. no. ANC-253-050; Ancell, Bayport, MN) was added before flow cytometry was performed. A flow cytometry instrument (EPICS ALTRA with HyPerSort cell sorting; Beckman Coulter, Fullerton, CA), located at the Human Gene Therapy Unit of Akdeniz University Hospitals and Clinics, was used to carry out flow cytometric analysis. Purified mouse IgG1 (MOPC 31C; cat. no. ANC-278-010; Ancell) was used as an isotype control. Monoclonal antibody to TRAIL (human) (cat. no. ALX-804-296-C100; Alexis Biochemicals) was applied, followed by polyclonal antibody to mouse IgG1 (R-PE) (cat. no. ALX-211-201-C050; Alexis Biochemicals), to reveal TRAIL expression on the cell surface.

TRAIL-R4 gene silencing by siRNA approach

Decoy receptor-2 (DcR2) siRNA experiments were conducted with DcR2 siRNA (cat. no. sc-35185; Santa Cruz Biotechnology, Santa Cruz, CA), siRNA transfection medium (cat. no. sc-36868), and siRNA transfection reagent (cat. no. sc-29528) in lung cancer cells as described by the manufacturer. Product sc-35185 is a pool of three siRNA sequences (to increase the silencing effect) designed for TRAIL-R4 mRNA silencing (GenBank accession number NM_003840). Lung cancer cells were plated in 24-well plates at a density of 2×10^5 cells per well after being resuspended in fresh RPMI 1640 containing 10% fetal bovine serum without antibiotics. Cells were then grown overnight to 40–50% confluency. The next day, siRNA–siRNA transfection reagent complex was prepared as a mixture of transfection solutions A and B (solution A: 1.8 μl of 10 μM siRNA mixed with 30 μl of siRNA transfection medium; solution B: 1.8 μl of siRNA transfection reagent

mixed with 7.25 μl of siRNA transfection medium), and incubated at room temperature for 20 min. The optimal dose of DcR2 siRNA needed to block TRAIL-R4 expression without serious cytotoxic effects was determined on the basis of a concentration gradient. Medium was removed from cells in the 24-well plate, and 300 μl of fresh growth medium (10% serum, without antibiotics) was added to each well. The transfection reagent complex was then added to the cells dropwise while gently rocking the plate. After transfection, cells were incubated for 30 hr at 37°C. After 30 hr, cells were infected with increasing MOIs of either Ad5hTRAIL viral vector or Ad-CMVLacZ at a constant MOI of 10,000 DNA particles/cell. Uninfected plus untransfected, uninfected plus transfected, and infected plus untransfected cells were used as controls. Transfection efficiency was originally determined with fluorescein-conjugated control siRNA-A (cat. no. sc-36869) under a fluorescence microscope. More than 95% of the cells were transfected by this procedure. Control siRNA-A (sc-37007) containing scrambled sequences that did not lead to the specific degradation of any cellular RNA was used as a negative control in siRNA experiments.

In vitro *siRNA synthesis*

Desalted DNA oligonucleotides encoding top- and bottom-strand target sequences (Table 1) were ordered from Integrated DNA Technologies (Coralville, IA). T7 promoter sequence (Table 1) was added at the 3′ ends of each primer for *in vitro* transcription. *In vitro* synthesis of siRNA was carried out with an AmpliScribe T7 high-yield transcription kit (Epicentre Biotechnologies, Madison, WI). Briefly, 1 μg of target oligonucleotide was mixed with 1 μg of T7 promoter oligonucleotide by heating at 95°C for 5 min, after which the heating block was switched off and allowed to cool slowly to 70°C. The tube was moved to a 37°C water bath for an additional 15 min to obtain annealed, double-stranded DNA (dsDNA). *In vitro* synthesis of siRNA was performed according to the manufacturer's protocol. The transcription mix included 1× T7 AmpliScribe buffer, 7.5 m*M* rNTPs, 10 m*M* dithiothreitol (DTT), T7 RNA polymerase, and 1 μg of dsDNA as a template. After incubation at 37°C for 2 hr, 1 U of RNase-free DNase I was added at 37°C for 15 min. To obtain small interfering double-stranded RNA, top- and bottom-strand RNAs generated in separate reactions were annealed by the following procedure. The two transcription reactions were mixed and heated at 95°C for 5 min. The heating block was switched off and allowed to cool slowly to 70°C. The reaction tube was then transferred to 37°C for an additional 15 min. siRNA was precipitated with 3 volumes of ethanol after adding 100 μl of nuclease-free water with 1 m*M* EDTA and 0.125 *M* LiCl.

Table 1. Primer Sequences for the Generation of DcR2 siRNA Oligonucleotides[a]

TRAIL-R4 siRNA-1	
Top:	GGATGGTCAAGGTCAGTAA
Bottom:	TTACTGACCTTGACCATCC
TRAIL-R4 siRNA-2	
Top:	CCCTATCACTACCTTATCA
Bottom:	TGATAAGGTAGTGATAGGG
TRAIL-R4 siRNA-3	
Top:	GCTTGGGAATGGTGTGAAA
Bottom:	TTTCACACCATTCCCAAGC

Abbreviations: DcR2, decoy receptor-2; siRNA, small interfering RNA; TRAIL-R4, tumor necrosis factor-related apoptosis-inducing ligand receptor-4.

[a]The primer 5′-TAATACGACTCACTATAG-3′ is annealed to all oligonucleotides to synthesize siRNAs.

TRAIL-R4 cDNA transfection and RNA interference assays

Monkey kidney fibroblast Cos-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum and penicillin–streptomycin mix, and human lung A549 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal calf serum and penicillin–streptomycin mix, at 37°C in a 5% CO_2 atmosphere. Cells were transfected with Lipofectamine Plus reagent (Invitrogen Life Technologies) according to the manufacturer's protocol. Cos-7 cells (2×10^5) were transfected with plasmid vector carrying human TRAIL-R4 cDNA (a gift of T.S. Griffith, University of Iowa, Iowa City, IA) alone, or in combination with *in vitro*-transcribed siRNAs for TRAIL-R4. Cells were transfected with 0.3 μg of human TRAIL-R4 cDNA expression vector and 1.5 μg of siRNA (1:5 ratio). The control group was transfected with 0.3 μg of TRAIL-R4 cDNA expression vector. To measure the silencing of endogenous TRAIL-R4 gene expression, the A549 human lung cancer cell line was transfected as described above with *in vitro*-transcribed siRNAs for TRAIL-R4, separately or in combination. Twenty-four hours after transfection, RNA was isolated with TRIzol reagent for subsequent real-time RT-PCR assay as described above.

Establishment of primary cell cultures from patients with lung carcinoma

Immediately after the surgical removal of lung carcinoma tissue from patients, the tissue was immersed in serum-free RPMI 1640 medium containing antibiotics, and kept cold while being transported to the laboratory. A microdissection technique was used to directly obtain samples from each of the tumors. Lung cancer tissue was then minced into small pieces with forceps and scissors in a sterile 10-cm Petri dish. Minced tissue samples were treated with collagenase type II (300 units/ml [cat. no. G6885]; Sigma, St. Louis, MO) in RPMI 1640 at 37°C for 2.5 hr with gentle stirring. After incubation, the cells were collected by gentle pipetting into a centrifuge tube. After centrifugation, the cells were washed twice with culture medium without FBS. After washing, the cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, insulin (15 μg/ml [cat. no. I1882]; Sigma), human epidermal growth factor (20 ng/ml [cat. no. E9644]; Sigma), and fibronectin (100 ng/ml [cat. no. F4759]; Sigma) at 37°C in a humidified 5% CO_2 incubator. In addition, two methods were used to eliminate possible fibroblast contamination in culture. The first was a partial trypsinization procedure in which a low concentration of trypsin solution (0.05%) was used. This protocol is based on the principle that fibroblasts are easier to detach than epithelial cells. The second approach involved the performance of a differential detachment technique. This protocol, based on the princi-

ple that fibroblasts attach to a dish surface faster than do epithelial cells, involves plating cells for a short period of time (2–6 hr), recovering unattached cells, and finally plating them into a new dish. The incubation period lasted several weeks before conducting our molecular assays.

Immunohistochemistry of TRAIL and its cognate receptors in patients with lung cancer

All primary antibodies were obtained from Alexis Biochemicals. The following primary antibodies (diluted 1:300) were deployed for the immunohistochemical analysis of lung specimens: Monoclonal antibody to TRAIL-R1 (human [HS101]; cat. no. ALX-804-297A-C100), polyclonal antibody to TRAIL-R2 (cat. no. ALX-210-743-C200), polyclonal antibody to TRAIL-R3 (human; cat. no. ALX-210-744-C200), and monoclonal antibody to TRAIL-R4 (human [HS402]; cat. no. ALX-804-299A-C100). The specificity of these primary antibodies was previously confirmed by Alexis Biochemicals. Specimens were immunostained in the absence of primary antibodies and these were used as negative controls. No immunostaining was done when primary antibodies were not used.

Immunohistochemical scoring of TRAIL and TRAIL receptors for patients with lung cancer

Slides of the specimens were analyzed by an independent pathologist (G.O.) who had no prior knowledge of the clinical data. Immunostaining scores were given on the basis of both the intensity and marker distribution (percentage of positively stained epithelial cells) in lung carcinoma as described previously (Sanlioglu *et al*., 2007). Intensity was scored as follows: 0, negative; 1, weak; 2, moderate; and 3, strong staining. Marker distribution was scored as follows: 0, less than 10%; 1, between 10 and 40%; 2, between 40 and 70%; and 3, more than 70% of the epithelial cells stained on the specimen. The final immunostaining score was obtained by adding the scores for both intensity and marker distribution for a given patient. SPSS 13.0 software for Windows (SPSS, Chicago, IL) was used for statistical analysis. Normality of the patient groups (adenocarcinoma [AC] and squamous cell lung carcinoma [SCC]) was tested by the Shapiro–Wilk method. None of the groups displayed a Gaussian distribution. For this reason, the significance of differences among the groups was determined by Friedman test. Later, the Wilcoxon signed ranks test with Bonferroni's correction was applied to compare paired groups of two.

RESULTS

First-generation adenoviral vectors efficiently transduce lung cancer cells

Before performing TRAIL transfer into lung cancer cells, the efficacy of the first-generation recombinant adenoviral vector transduction of lung cancer cells was revealed with an adenoviral vector encoding enhanced green fluorescent protein (AdEGFP). The main rationale for performing these assays was to determine the optimal dose of adenovirus to conduct gene delivery. The efficacy of viral infection was monitored by fluorescence microscopy, and the transduction results were quantitatively analyzed by flow cytometry 48 hr after infection. Almost 100% of the cells were efficiently transduced with AdEGFP at an MOI of 5000 DNA particles/cell 48 hr after infection (data not shown).

A549 lung carcinoma cell line is resistant to adenoviral delivery of hTRAIL

Despite the fact that the death ligand TRAIL exerts anticancer properties, the observed TRAIL resistance of cancer cells has critically challenged the use of TRAIL as a gene therapy agent. To find out whether A549 lung cancer cells are resistant to TRAIL, an adenoviral vector encoding hTRAIL (Ad5hTRAIL) or β-galactosidase (AdCMVLacZ) was transferred at increasing doses into A549 lung cancer cells. Infection with neither Ad5hTRAIL (MOI of up to 10,000 DNA particles/cell) nor AdCMVLacZ (control, data not shown) vector reduced the amount of viable cells 48 hr after infection, as depicted in Fig. 1A. To rule out the possibility that the failure to observe any cytotoxic effect was due to the lack of TRAIL expression from the vector, a flow cytometric analysis was performed with A549 cells infected with Ad5hTRAIL (Fig. 1B). This assay demonstrated that significant levels of TRAIL overexpression were achieved through infection of A549 cells with Ad5hTRAIL.

TRAIL receptor expression profile of A549 lung cancer cells

Despite extensive efforts to determine the mechanism of TRAIL resistance in cancer cells, no connection between the expression pattern of TRAIL receptors and TRAIL sensitivity has been demonstrated (Griffith *et al*., 1998). Quantitative real-time RT-PCR assays were carried out with primer–probe sets specifically designed to detect each of the TRAIL receptors in A549 lung cancer cells (Fig. 2A). Although all TRAIL receptors were present in A549 lung cancer cells, TRAIL-R2 death receptor gene expression was the highest among the four. TRAIL-R1 and TRAIL-R4 receptor gene expression was evident at similar levels, and TRAIL-R3 decoy receptor gene expression was relatively low compared with the other three receptors. Conventional flow cytometric analysis was conducted with antibodies specific to each of the TRAIL receptors, because mRNA expression inside the cell may not necessarily correlate with protein expression on the cell surface. Results of this assay suggested that all four TRAIL receptors were expressed on the surface of A549 lung cancer cells (Fig. 2B). Substantial levels of TRAIL-R4 decoy receptor protein expression were evident on the surface of A549 lung cancer cells. TRAIL-R3 decoy receptor protein expression was lowest among the four but was, nonetheless, still detectable on the cell surface.

DcR2 siRNA approach sensitizes A549 lung cancer cells to TRAIL

A DcR2 siRNA approach was used to attenuate or block TRAIL-R4 decoy receptor expression in A549 lung cancer cells. Downregulation of TRAIL-R4 protein expression following DcR2 siRNA administration was confirmed by flow cytometry (Fig. 3A). Expression of none of the TRAIL receptors was affected when control siRNA-A was used as a negative

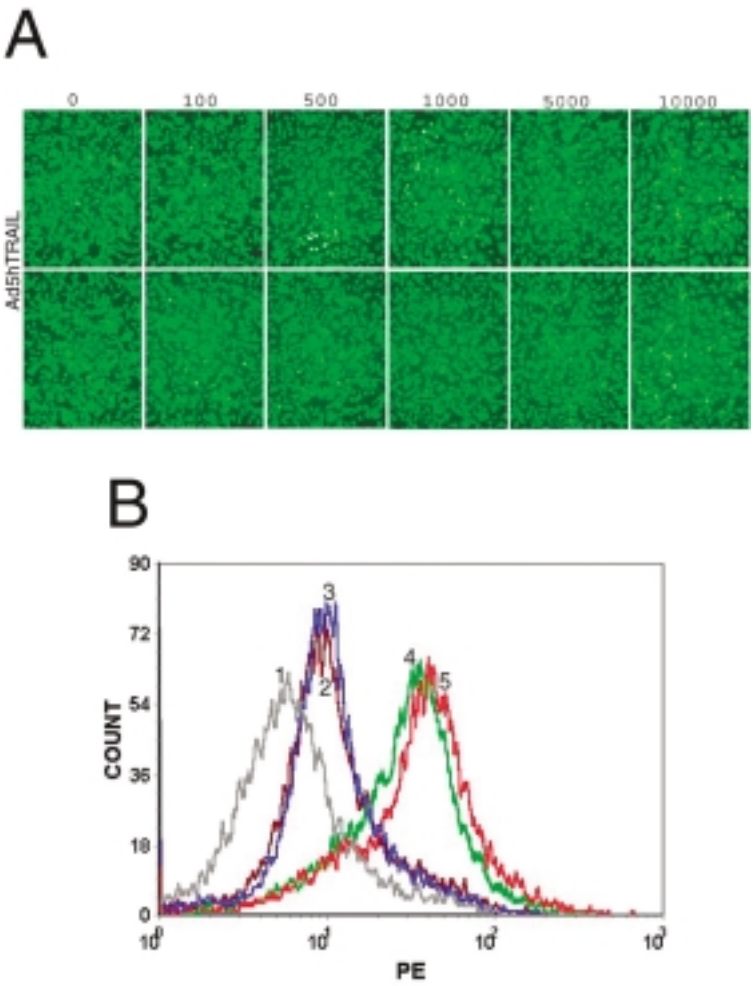

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FIG. 1. A549 lung cancer cells exhibit TRAIL resistance. (**A**) Ad5hTRAIL vector was transferred into A549 lung cancer cells at increasing doses as described in Materials and Methods. An Invitrogen Molecular Probe LIVE/DEAD viability/toxicity kit was used to detect viable cells 48 hr after infection. The viral doses applied are presented as MOI values (DNA particles per cell) above each panel. Duplicate samples are given for each infection. (**B**) Flow cytometric analysis of hTRAIL expression in A549 cells infected with Ad5hTRAIL. Conditions for infection were as follows: 1, unstained; 2, uninfected (secondary antibody alone); 3, infected with AdLacZ; 4, infected with Ad5hTRAIL at an MOI of 5000 DNA particles/cell; 5, infected with Ad5hTRAIL at an MOI of 10,000 DNA particles/cell.

control for the transfection instead of DcR2 siRNA. Then, either Ad5hTRAIL or AdCMVLacZ (negative control) infection was performed at increasing doses after DcR2 siRNA administration in A549 lung cancer cells. Proportions of live and dead cells were determined 48 hr after infection. Although A549 lung cancer cells were previously shown to be resistant to TRAIL, downregulation of TRAIL-R4 decoy receptor expression resulted in the sensitization of A549 lung cancer cells to Ad5hTRAIL (Fig. 3B). No such effect was observed when A549 lung cancer cells were infected with AdCMVLacZ virus (Fig. 3B). TRAIL-R4 decoy receptor expression was reduced by 85 ± 7% after DcR2 siRNA administration as demonstrated by flow cytometry. In addition, quantitative real-time RT-PCR analysis was performed in order to determine the level of TRAIL-R4 RNA after DcR2 siRNA administration. This assay indicated a 75 ± 5% decrease in TRAIL-R4 RNA levels.

Because the DcR2 siRNA kit (cat. no. sc-35185) from Santa Cruz Biotechnology consists of three siRNA constructs in combination developed against TRAIL-R4 mRNA, custom synthesis of individual siRNA constructs was performed by a completely different approach as described in Materials and Methods in order to check the efficacy of individual siRNAs for their inhibitory effect on TRAIL-R4 expression (Table 1). As a first step, monkey kidney fibroblast Cos-7 cells were transfected with TRAIL-R4-encoding cDNA alone or in combination with individual *in vitro*-transcribed siRNAs. A nonhuman cell line was chosen for the proof-of-principle experiments because the lack of TRAIL-R4 expression on these cells provided a proper control. As seen in Fig. 4A, all three double-stranded siRNAs effectively downregulated TRAIL-R4 mRNA expression on Cos-7 cells. After confirming that *in vitro*-transcribed siRNAs effectively interfered with the expression of ectopically expressed TRAIL-R4 the silencing effect of the same siRNAs was examined in A549 cells. As demonstrated in Fig. 4B, siRNA-3 and the combination of three siRNAs were effective in reducing TRAIL-R4 expression to an extent greater than 80% (Fig. 4B). The level of silencing was similar to the result obtained with the DcR2 siRNA kit (cat. no. sc-35185; Santa Cruz Biotechnology). Infection with Ad5hTRAIL at an MOI of 5000 DNA particles/cell resulted in a 65 ± 6% decrease in cell viability after transfection with the siRNA cocktail containing three oligonucleotides in combination. However, some differences were also observed between the two sets of experiments. This difference, between Cos-7 and A549 cells, can be attributed to the experimental setup consisting of an overexpression scenario (Cos-7 cells) versus endogenous levels of TRAIL-R4 mRNA (A549

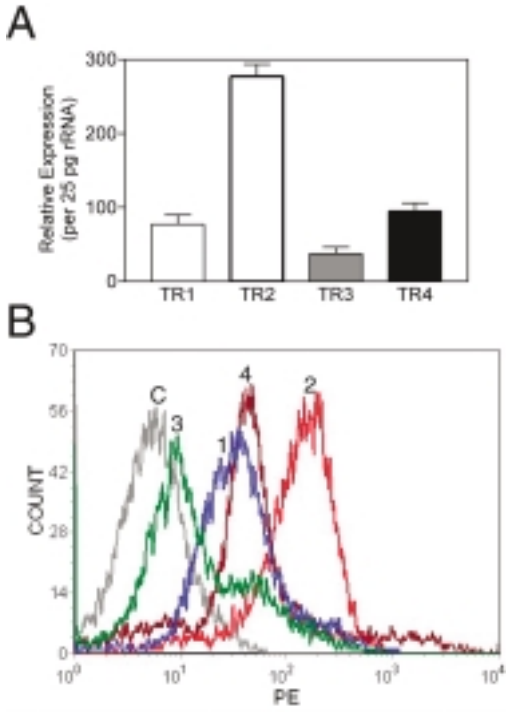

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FIG. 2. TRAIL receptor profile of A549 lung cancer cells. (**A**) A real-time RT-PCR assay was performed to quantify the amounts and types of TRAIL receptor expressed on A549 lung cancer cells. Ribosomal RNA primers and probes were included in each TaqMan reaction as an internal control. Relative expression per 25 pg of rRNA is provided on the *y* axis. (**B**) Surface TRAIL receptor profile of A549 lung cancer cells. Conventional flow cytometric analyses were performed to detect the surface expression profile of TRAIL receptors as explained in Materials and Methods. Peak C, isotype control staining; peaks 1–4, TRAIL-R1 through TRAIL-R4, respectively. A total of 10^4 A549 lung cancer cells was gated for each histogram. This assay was repeated three times, but only one representative assay is shown for clarity.

cells). Nevertheless, in both sets of experiments, generated siRNAs substantially reduced the expression of TRAIL-R4.

TRAIL-sensitive H411 and HBE lung carcinoma cell lines express all TRAIL receptors except TRAIL-R4

To strengthen the correlation between the decoy receptor gene expression and TRAIL resistance observed in A549 cells, two other non-small cell lung carcinoma cell lines were screened. Interestingly, neither H411 nor HBE lung carcinoma cells exhibited detectable levels of TRAIL-R4 decoy receptor expression on the cell surface as demonstrated by flow cytometric analysis (Fig. 5). Ad5hTRAIL infection alone at an MOI of 5000 DNA particles/cell resulted in 80% reduction in the viability of HBE cells and 73% reduction in the viability of H411 cells. Obviously, surface TRAIL-R3 decoy receptor expressed on both of these cell lines was not able to protect the cells from TRAIL-induced cytotoxicity. These results may suggest that in the absence of TRAIL-R4 expression on the cell surface, TRAIL death receptors are the main modulators of TRAIL sensitivity in lung cancer cells.

TRAIL-resistant primary lung carcinoma cells display high levels of TRAIL-R4 decoy receptor expression on the cell surface

To solidify the connection between the expression pattern of TRAIL receptors and TRAIL sensitivity, primary cell cultures were established from three patients with non-small cell lung carcinoma (NSCLC). Two of these patients had squamous cell lung carcinoma (SCC) and one had adenocarcinoma (AC). SCC cases were diagnosed on the basis of immunohistochemical staining of paraffin-embedded sections (Fig. 6A). A real-time RT-PCR assay conducted with a primary cell culture established from the first patient with SCC indicated that although all four TRAIL receptors were expressed in this patient, TRAIL-R4 decoy receptor expression was the highest, based on mRNA levels (Fig. 6B). In addition, only TRAIL-R4 decoy receptor expression was evident on the surface of primary lung cancer cells

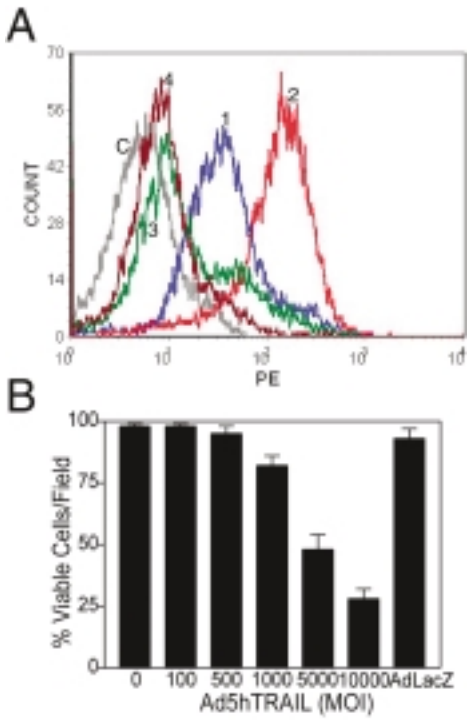

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FIG. 3. Downregulation of TRAIL-R4 expression sensitizes A549 lung cancer cells to TRAIL. (**A**) DcR2 siRNA approach targeting TRAIL-R4 expression in A549 lung cancer cells. Other TRAIL receptor expressions are shown as controls. C represents isotype control staining. Each histogram represents 10^4 gated A549 lung cancer cells. The number above each peak corresponds to a specific TRAIL receptor (1 = TRAIL-R1, etc.). One of three independent flow assays is given. Downregulation of TRAIL-R4 decoy receptor gene expression knocks out TRAIL resistance in A549 lung cancer cells (**B**). The DcR2 siRNA approach was conducted as described in Materials and Methods. AdLacZ in (**B**) refers to AdCMVLacZ virus infected at an MOI of 10,000 DNA particles/cell into A549 cells after DcR2 siRNA transfection in place of Ad5hTRAIL virus. Data represent means and SEM of three independent assays ($n = 6$).

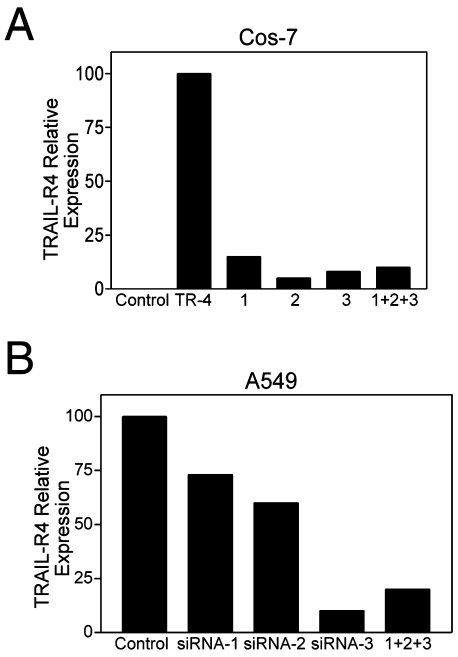

FIG. 4. *In vitro*-synthesized siRNA constructs downregulate TRAIL-R4 expression in Cos-7 cells (**A**) and A549 cells (**B**). siRNA constructs (siRNA-1 to siRNA-3), displayed on the *x* axis, were synthesized as described in Materials and Methods. Except for the control samples, Cos-7 cells were transfected with TRAIL-R4 cDNA before the siRNA assay, as shown in (**A**). TRAIL-R4 expression levels were detected by real-time RT-PCR assay after transfection. Treatment conditions are provided on the *x* axis. A 1.5-μg amount of each siRNA was used in transfection with the exception of combined siRNA administration (column 1+2+3), in which 0.5 μg of each siRNA construct (total, 1.5 μg) was used. Experiments were repeated twice to confirm the observations; data from only one representative assay are provided.

as detected by flow cytometry (Fig. 6C). No TRAIL receptor other than TRAIL-R4 was expressed at detectable levels on the surface (data not shown for clarity). As expected, primary lung carcinoma cells were completely resistant to adenoviral delivery of TRAIL up to an MOI of 10,000 DNA particles/cell (data not shown). Furthermore, the DcR2 siRNA approach downregulated surface TRAIL-R4 expression (peak marked with an asterisk in Fig. 6C) but still failed to sensitize cells to adenoviral delivery of TRAIL. Although the lack of death receptor gene expression can also account for TRAIL resistance in this particular case, expression of high levels of TRAIL-R4 decoy receptor on the cell surface may imply that this phenotype is not restricted only to the A549 lung carcinoma cell line. Real-time RT-PCR analysis of a primary cell culture established from the second patient with SCC indicated, on the basis of mRNA levels, that all four TRAIL receptors were expressed (Fig. 7A). Flow cytometric analysis demonstrated that higher levels of TRAIL-R4 decoy receptor were expressed on the cell surface compared with TRAIL death receptors TRAIL-R1 and TRAIL-R2. But TRAIL-R3 decoy receptor expression was not detectable on the surface. DcR2 siRNA transfection, but not siRNA-A, downregulated (86%) surface TRAIL-R4 expression (Fig. 7B). Later, infection with Ad5hTRAIL, but not Ad-CMVLacZ, at an MOI of 10,000 DNA particles/cell, resulted in TRAIL sensitization (42 $\pm$ 5%) of DcR2 siRNA-transfected primary lung cancer cells (data not shown). The third patient was diagnosed with AC. The primary cell culture established from this patient manifested higher levels of TRAIL-R2 and TRAIL-R4 mRNA expression compared with TRAIL-R1, as demonstrated by real-time RT-PCR assays (Fig. 8A). In this case, TRAIL-R3 decoy receptor mRNA expression was not detectable. Accordingly, only TRAIL-R2 and TRAIL-R4 receptors were evident on the cell surface as shown by flow cytometric analysis (Fig. 8B). Next, surface TRAIL-R4 expression was downregulated by the DcR2 siRNA approach (76%), but not by administration of siRNA-A, as presented in Fig. 8B. This resulted in TRAIL sensitization of lung cancer cells treated with Ad5hTRAIL, but not with AdCMVLacZ, at an MOI of 10,000 DNA particles/cell to an extent greater than 68 $\pm$ 6% (data not shown).

NSCLC patients display higher levels of TRAIL-R4 expression compared with other TRAIL receptors

To investigate the *in vivo* relevance of the preceding findings, TRAIL receptor profiles of patients with lung cancer were examined by immunohistochemical analysis of paraffin-embedded sections obtained from patients either with AC or SCC (Fig. 9A). On the basis of blinded scoring as described in

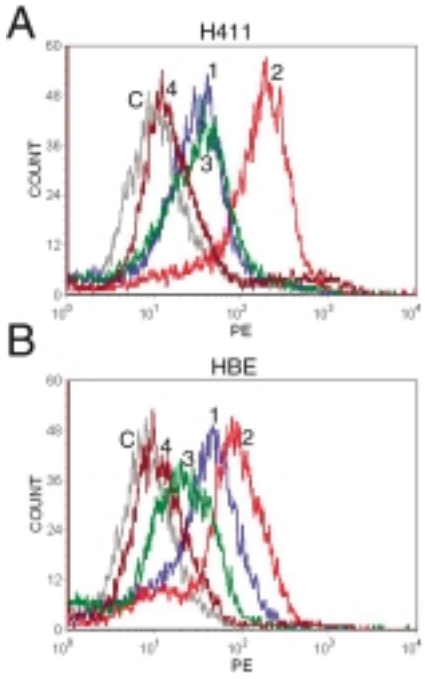

FIG. 5. TRAIL-sensitive lung cancer cell lines (H411 and HBE) displayed undetectable levels of TRAIL-R4 expression on the cell surface. Flow cytometric analysis and Ad5hTRAIL infections were performed as described in Materials and Methods. A total of 10^4 lung cancer cells was gated for each histogram. Peak C, isotype control staining; peaks 1–4, TRAIL-R1 through TRAIL-R4, respectively. Assays were performed in duplicate to confirm the results.

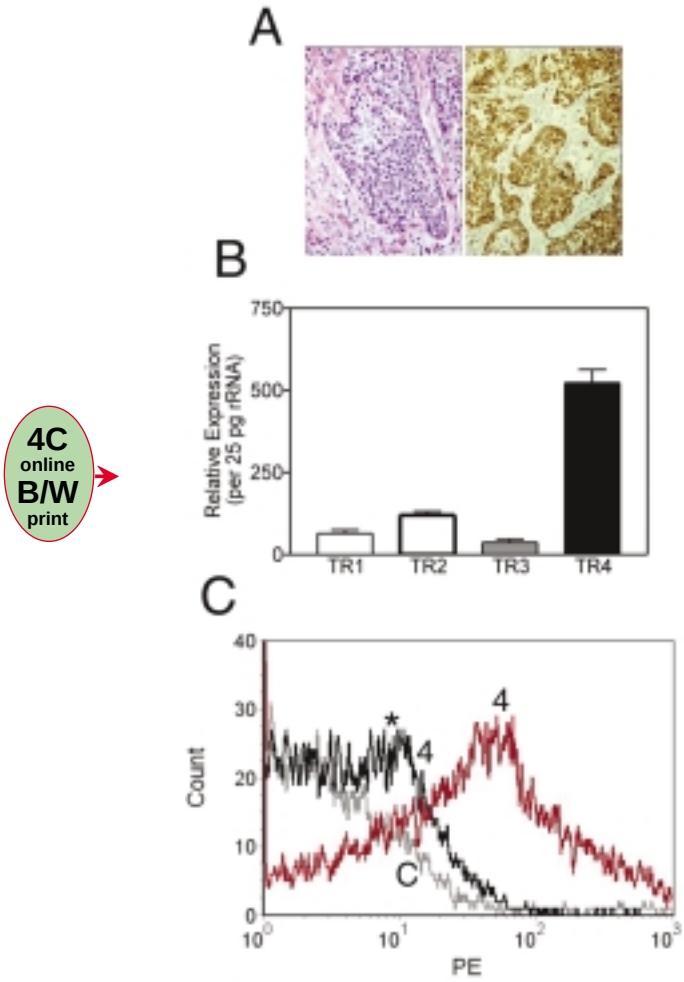

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FIG. 6. Decoy receptor TRAIL-R4 is the main receptor expressed in a primary cell culture from a patient with SCC. The primary lung cell culture was prepared as described in Materials and Methods. (**A**) H&E staining (*left panel*) of lung cancer tissue and high molecular weight keratin-positive cells (*right panel*), which indicate squamous cell lung carcinoma. (**B**) Real-time RT-PCR assay displaying intracellular TRAIL receptor composition at the mRNA level. (**C**) Flow cytometric analysis of the same primary lung carcinoma cell culture. A total of 10^4 lung cancer cells was gated for each histogram. The results of one of two independent assays is shown. Peak C, isotype control staining; peak 4, TRAIL-R4; peak 4*, DcR2 siRNA-transfected TRAIL-R4 sample.

Materials and Methods, both SCC and AC patients displayed higher levels of TRAIL-R4 expression compared with other types of TRAIL receptors (Fig. 9B).

DISCUSSION

It has been reported that at least 50% of human tumors exhibit resistance to TRAIL (Griffith and Lynch, 1998). In accordance with this, two different hypotheses are asserted to explain TRAIL resistance. The first hypothesis suggests the presence of decoy receptors that compete for binding to TRAIL (Sheridan *et al*., 1997; Srivastava, 2001). According to this theory, these receptors either dilute out TRAIL ligands (such as TRAIL-R3) or supply antiapoptotic signals (like TRAIL-R4) to cells (Chaudhary *et al*., 1997; Pan *et al*., 1997). However, RNase protection assays did not reveal any connection between TRAIL sensitivity and the expression of TRAIL-R1, TRAIL-R2, and TRAIL-R3 in lung cancer cell lines (Kagawa *et al*., 2001). Although this particular study also demonstrated that A549 lung cancer cells were relatively resistant to a TRAIL-mediated gene therapy approach, TRAIL-R4 expression in the A549 cell line was not examined. The second hypothesis claims the presence of apoptosis-inhibitory molecules that counteract TRAIL-mediated apoptosis (French and Tschopp, 1999a). For example, the level of c-FLIP (cellular FLICE [FADD-like interleukin-1β-converting enzyme]-inhibitory protein) expression has previously been blamed for TRAIL resistance in cancer cells (French and Tschopp, 1999b). However, screening of NSCLC cell lines by Western blotting did not reveal any correlation between the expression pattern of c-FLIP and TRAIL resistance (Frese *et al*., 2002). Contrary to expectation, TRAIL-sensitive cell lines (NCI-H358) expressed higher levels of c-FLIP, whereas low levels of c-FLIP were observed in TRAIL-resistant cell lines (A549, Calu1, and SkLu1). Accordingly, it

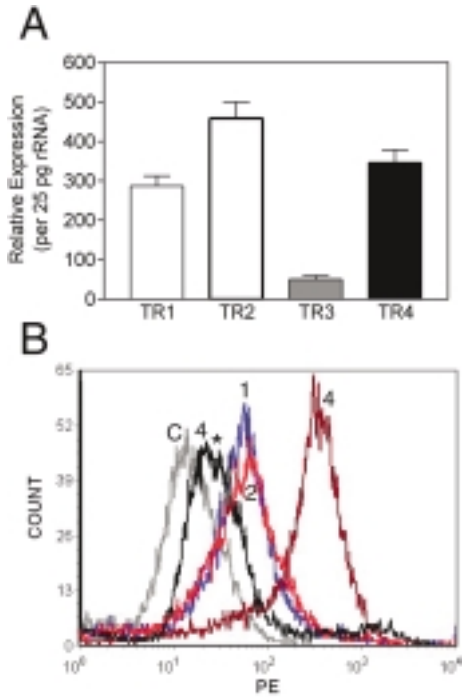

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FIG. 7. Quantitative RT-PCR (**A**) and flow cytometric analysis (**B**) of a primary cell culture established from the second patient with SCC. Relative expression per 25 pg of rRNA is given on the *y* axis for the real-time PCR assay. Data represent means and SEM of two independent assays ($n = 6$). Flow cytometric analyses were conducted after transfection either with DcR2 siRNA or siRNA-A. A total of 10^4 lung cancer cells was gated for each histogram. The results of one of two independent assays is shown. Peak C, isotype control staining; peaks 1, 2, and 4, TRAIL-R1, TRAIL-R2, and TRAIL-R4, respectively; peak 4*, DcR2 siRNA-transfected TRAIL-R4 sample.

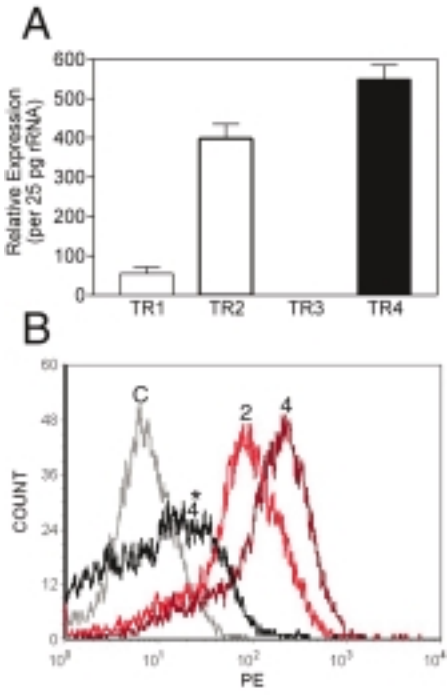

FIG. 8. TRAIL receptor profile of a primary cell culture established from a patient with AC. (**A**) The level of TRAIL receptor transcripts as assessed by real-time RT-PCR analysis. The *y* axis shows relative expression per 25 pg of rRNA. Data represent means and SEM of two independent assays ($n = 6$). (**B**) Flow cytometric analysis indicating the surface TRAIL receptor expression pattern revealed after transfection either with siRNA-A or DcR2 siRNA. A total of 10^4 lung cancer cells was gated for each histogram. The results of only one of two independent experiments are shown. Peak C, isotype control staining; peaks 2 and 4, TRAIL-R2 and TRAIL-R4, respectively; peak 4*, DcR2 siRNA-transfected TRAIL-R4 sample.

was concluded that mechanisms other than the level of c-FLIP expression might be responsible for TRAIL resistance in NSCLCs.

Although efforts have been made to elucidate the molecular mechanism of TRAIL resistance, no direct correlation between the expression pattern of TRAIL receptors and TRAIL resistance has been reported (Kagawa *et al*., 2001). To assess the level of TRAIL receptor gene expression in A549 lung cancer cells, quantitative real-time RT-PCR assays were performed. This assay demonstrated that all four TRAIL receptors were expressed in A549 lung cancer cells, and that the expression level of TRAIL-R2 death receptor was the highest among the four. Because mRNA levels inside the cell may not correlate with protein expression on the cell surface (the latter being more relevant for TRAIL sensitivity), we performed flow cytometric analysis. We found that although all TRAIL receptors were expressed on the cell surface, there were substantial levels of TRAIL-R4 decoy receptor protein expression on the surface of A549 lung cancer cells. Interestingly, it has previously been reported that TRAIL-R4 overexpression protected target cells from TRAIL-induced cytotoxicity (Degli-Esposti *et al*., 1997). This report claimed that transient TRAIL-R4 expression protected cells from apoptosis by acting both as a decoy receptor and as an antiapoptotic signal provider. In addition, adenovirus-mediated *p53* delivery upregulated TRAIL-R4 mRNA expression, delaying TRAIL-, p53-, and TRAIL-R2-dependent colon cancer apoptosis (Meng *et al*., 2000). To reveal the possible role of TRAIL-R4 expression in the constitution of TRAIL resistance, a DcR2 siRNA approach was applied before the infection of A549 lung cancer cells with Ad5hTRAIL. Intriguingly, downregulation of TRAIL-R4 expression sensitized TRAIL-resistant A549 lung cancer cells to TRAIL. In addition, two other NSCLC cell lines (H411 and HBE), which expressed undetectable levels of TRAIL-4 decoy receptors on the cell surface, were tested for sensitivity to the Ad5hTRAIL vector. Despite the expression of TRAIL-R3 decoy receptor on the surface these two cell lines were relatively sensitive to TRAIL overexpression. To solidify the connection between TRAIL-R4 expression and TRAIL resistance, primary cell cultures were established from three patients with NSCLC. All primary cell cultures expressed high levels of TRAIL-R4 decoy receptor on the cell surface and all were resistant to adenoviral delivery of TRAIL. DcR2 siRNA, but not siRNA-A, transfection sensitized these primary cell cultures to the cytotoxic effects of TRAIL as long as a death receptor was present on the cell surface. All these results may suggest that TRAIL-R4 expression on the cell

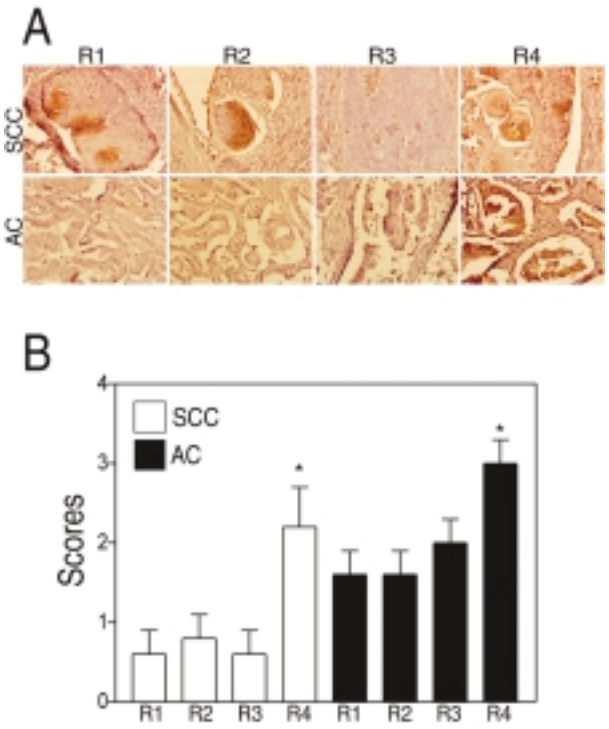

FIG. 9. Immunohistochemical analysis of TRAIL and TRAIL receptors in patients with lung cancer. Five patients with SCC and five other patients with AC were analyzed in terms of TRAIL and TRAIL receptor gene expression, using immunohistochemistry. Bright-field images are shown in (**A**). Immunohistochemical staining scores are provided in (**B**). Procedures for immunohistochemical staining and scoring and statistical analyses are described in Materials and Methods. *$p < 0.05$, compared with other TRAIL receptors.

surface drastically influences TRAIL sensitivity in cancer cells. Consequently, testing of primary lung cancer cells established from patients as well as immunohistochemical analysis of 10 other patients with NSCLC demonstrated that high-level TRAIL-R4 decoy receptor expression was a common phenotype observed in patients with lung cancer. These results suggest that high levels of TRAIL-R4 decoy receptor expression should be accounted for when treating NSCLC patients with Ad5hTRAIL as the sole treatment modality.

In conclusion, this study demonstrates that surface TRAIL-R4 decoy receptor expression correlated well with the TRAIL resistance phenotype in lung cancer cells. Furthermore, in addition to NF-κB inhibition (Sanlioglu *et al*., 2006), the alteration of TRAIL receptor profiles of cancer cells by a DcR2 siRNA approach is a novel way of bypassing the TRAIL resistance encountered in cancer cells.

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