

Combination of TRAIL Gene Therapy and Chemotherapy Enhances Antitumor and Antimetastasis Effects in Chemosensitive and Chemoresistant Breast Cancers

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We recently found that breast cancer cell lines that are resistant to chemotherapy or to the recombinant TRAIL protein are susceptible to TRAIL gene therapy. However, it is unclear whether a combination of TRAIL gene therapy and chemotherapy will have enhanced antitumor activity or can be used for the treatment of metastasis. In this study, we investigated the combined effect of TRAIL gene therapy and chemotherapeutic agents, including doxorubicin, paclitaxel, vinorelbine, gemcitabine, irinotecan, and floxuridine, in different breast cancer cell lines. In all the cell lines tested, including a breast cancer cell line that is resistant to chemotherapy, the combination of TRAIL gene therapy and cytotoxic agents had either a synergistic or an additive effect. An *in vivo* study showed that aerosolized administration of an adenovector expressing the GFP-TRAIL fusion protein from the human telomerase reverse transcriptase promoter (designated Ad/gTRAIL) also decreased the number of lung metastases from both doxorubicin-sensitive and doxorubicin-resistant breast cancer cell lines. The combination of TRAIL gene therapy and chemotherapy resulted in a further reduction of lung metastatic nodules with minimal toxicity. These results suggest that a combination of TRAIL gene therapy and chemotherapy is effective in the treatment of metastatic diseases.

Key Words: drug synergism, drug resistance, aerosol drug therapy, lung, metastasis

INTRODUCTION

Despite the advent of many new therapeutic strategies and agents, more than 40,000 American women still die of breast cancer each year [1,2]. Adding to this situation is the fact that, although doxorubicin and paclitaxel are effective against some breast cancers [3,4], the anticancer treatment is frequently discontinued because of intolerable toxicity and/or the development of drug resistance. Consequently, the treatment of breast cancers in advanced stages is limited to being palliative. Thus, there is an urgent need for new therapeutics to be developed for the treatment of breast cancer, especially for metastatic breast cancers that are resistant to conventional therapy.

A major mode of resistance to antitumor therapy is insensitivity to apoptosis induction [5-7]. Apoptosis, a genetically regulated mechanism of cell death, is con-

trolled by a variety of genes, some with proapoptotic functions and others with antiapoptotic functions. The relative balance of these competing activities determines cell fate [8]. One possible new strategy in the treatment of breast cancer is to combine chemotherapeutic agents and proapoptotic genes that may trigger apoptosis at different levels or by different pathways, thereby enhancing apoptosis induction and resulting in a better outcome.

In one such effort, we and others recently found that the direct introduction of the TRAIL or GFP-TRAIL fusion gene into cancer cells can induce apoptosis and apoptotic bystander effects in breast, lung, colon, ovary, and prostate cancer cells [9-12]. Specifically, both doxorubicin-sensitive and doxorubicin-resistant breast cancer cells remained susceptible to treatment with an adenovector expressing the GFP-TRAIL fusion gene from the human telomerase reverse transcriptase promoter (designated Ad/

gTRAIL). The intratumoral injection of Ad/gTRAIL resulted in complete tumor regression in about 50% of the animals bearing doxorubicin-sensitive and doxorubicin-resistant breast cancer xenografts [12], suggesting that Ad/gTRAIL is a potent antitumor agent for both chemosensitive and chemoresistant tumors. Moreover, breast cancer cell lines that are resistant to the recombinant TRAIL protein (up to 800 ng/ml) are susceptible to TRAIL gene therapy [12]. Furthermore, transgene expression and apoptosis induction by Ad/gTRAIL was minimal in normal human fibroblasts, normal human primary hepatocytes, mammary epithelial cells, and ovary epithelial cells in culture [10–12]. However, it is unclear whether a combination of Ad/gTRAIL treatment and chemotherapy will lead to an enhanced cell killing effect in breast cancer cells and whether Ad/gTRAIL alone or in combination with chemotherapy is effective for the treatment of lung metastasis. In this study, we investigated the combined effects of Ad/gTRAIL and chemotherapy in the treatment of breast cancers and particularly metastatic breast cancer. Our results showed that combining Ad/gTRAIL with cytotoxic agents had synergistic or additive effects in breast cancer cell lines, including chemoresistant cells. In addition, the aerosol administration of Ad/gTRAIL in a complex with hydrocortisone and protamine in combination with chemotherapy significantly suppressed lung metastasis derived from breast cancer.

RESULTS

Combined Effects of Ad/gTRAIL and Doxorubicin in Vitro in Breast Cancer Cells

We tested the combined effects of Ad/gTRAIL and doxorubicin in four human breast cancer cell lines: MDA-MB-231, MDA-MB-453, MDA-MB-468, and MCF7. We treated the cells with Ad/gTRAIL in a series of multiplicities of infection (m.o.i.) ranging from 125 to 8000, with doxorubicin in a series of concentrations ranging from 0.0625 to 4.0 μ M, or with both. Mock-infected cells and cells treated with Ad/CMV-GFP under the same conditions as Ad/gTRAIL were used as controls. Cell viability was measured by the 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-(2H)-tetrazolium-5-carboxanilide (XTT) assay. Combined effects were analyzed using isobolograms as described previously [13]. A combination of Ad/gTRAIL and doxorubicin had a supra-additive effect in MDA-MB-231 and MDA-MB-468 cells and an additive effect in MDA-MB-453 and MCF7 cells (Fig. 1A). The observed data points for a combination fell mainly below the envelope (showing synergism) in MDA-MB-231 and MDA-MB-468 cells and within the envelope (showing additivity) in MDA-MB-453 and MCF7 cells. Flow cytometry assays also showed a dramatic increase in apoptosis after combination therapy when the m.o.i. for adenovectors was set at 1000 viral particles and the concentration of doxorubicin was set at 0.5 μ g/ml (Fig. 1B). Combined treatment with Ad/gTRAIL and doxo-

rubicin significantly increased the numbers of apoptotic cells in MDA-MB-231 and MDA-MB-468 cells. In MDA-MB-453 and MCF7 cells, the combination of Ad/gTRAIL and doxorubicin was more efficacious than Ad/gTRAIL or doxorubicin alone but not to a statistically significant level. Together, these results suggest that the combination of Ad/gTRAIL and doxorubicin has synergistic or additive effects in these cancer cell lines.

Combined Effects of Ad/gTRAIL and Other Chemotherapeutic Agents on Doxorubicin-Resistant Cancer Cells

To test whether a combination of Ad/gTRAIL and cytotoxic agents can also achieve enhanced cell-killing effects in chemoresistant breast cancer cells, we evaluated such combinations in doxorubicin-resistant 231/ADR breast cancer cells. This chemoresistance is highlighted by the fact that the doxorubicin concentration required for inhibiting 50% of cells (IC_{50}) is 1.14 μ M for parental MDA-MB-231 cells but is 97.6 μ M for 231/ADR cells [12]. Moreover, the IC_{50} for paclitaxel, vinorelbine, irinotecan, floxuridine, and gemcitabine is higher to various degrees for 231/ADR cells than for parental MDA-MB-231 cells, suggesting that 231/ADR cells are also relatively resistant to many other chemotherapy drugs [12]. We evaluated the combined effects of Ad/gTRAIL plus paclitaxel, vinorelbine, gemcitabine, irinotecan, and floxuridine in MDA-MB-231 and 231/ADR cells using an XTT assay and isobolograms, as described under Materials and Methods. In parental MDA-MB-231 cells, synergism was observed with the combination of Ad/gTRAIL and gemcitabine, whereas only additivity was observed with the combination of Ad/gTRAIL plus paclitaxel, vinorelbine, irinotecan, or floxuridine. In contrast, in 231/ADR cells synergism was observed with the combination of Ad/gTRAIL and paclitaxel, vinorelbine, irinotecan, or gemcitabine (Fig. 2). Similar to the results seen in MDA-MB-231 cells, the combination of Ad/gTRAIL and floxuridine had an additive effect in 231/ADR cells. However, combination of Ad/gTRAIL and doxorubicin did not resensitize 231/ADR cells to doxorubicin (data not shown).

Ad/gTRAIL Alone or in Combination with Doxorubicin or Paclitaxel for Treatment of Lung Metastases from Breast Cancer

We also tested whether Ad/gTRAIL alone or in combination with chemotherapy can be used for the treatment of lung metastases from breast cancer. Because the systemic administration of an adenovector results mainly in the transduction of liver tissue, we used aerosolized vector administration to deliver the gene to the lung. Our recent study showed that adenovectors in a complex with protamine and hydrocortisone can significantly increase

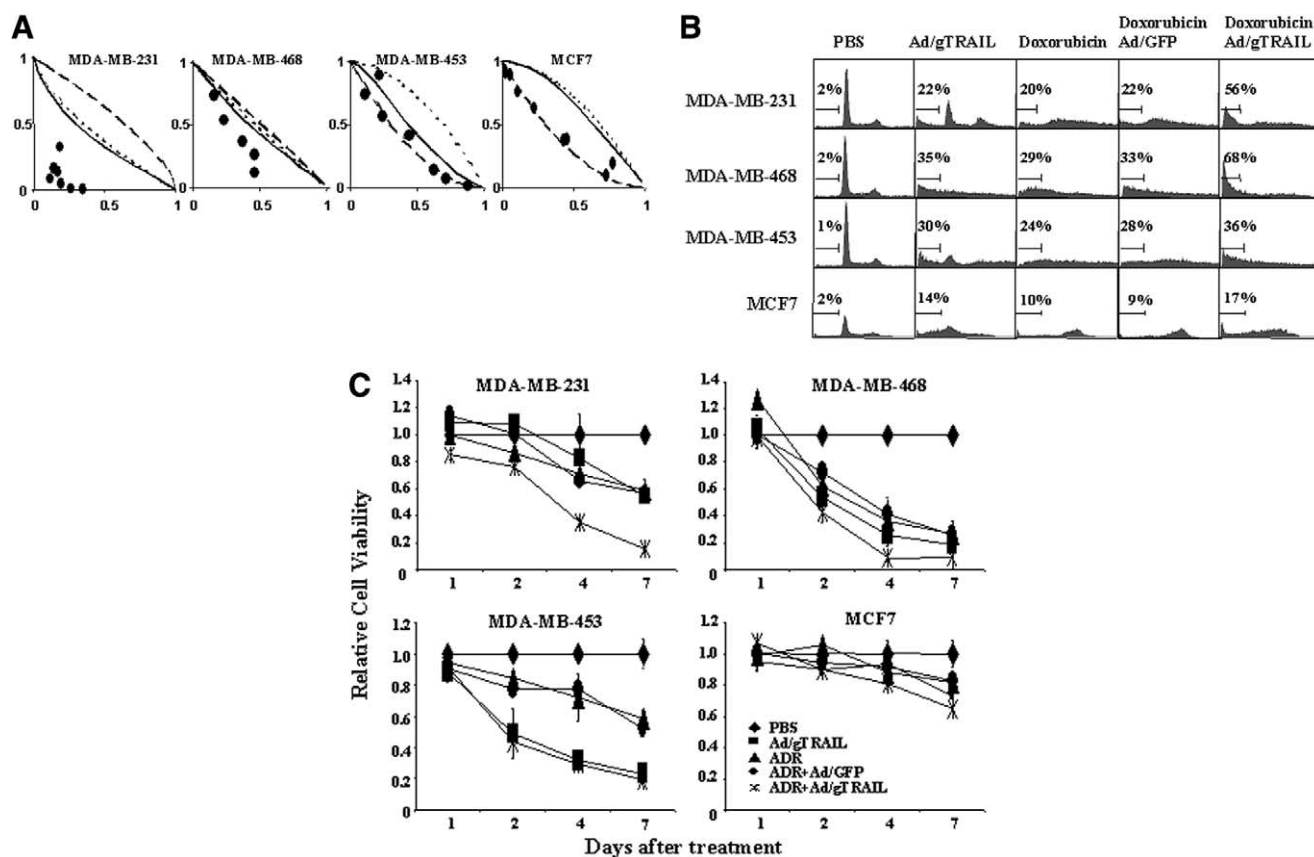


FIG. 1. Effects of Ad/gTRAIL plus doxorubicin *in vitro* in breast cancer cells. (A) IC_{50} isobolograms of the combined effects of Ad/gTRAIL and doxorubicin in different breast cancer cell lines. The x and y axes represent equieffect doses for 50% cell killing by chemotherapeutic agent and Ad/gTRAIL, respectively [13,38]. The observed data points are indicated by the filled symbols. (B) Flow cytometry analysis of transgene expression and apoptosis induction. The cells were harvested 96 h after treatment. Levels of apoptotic cell death were determined by FACS, which yielded the percentage of apoptotic sub-G1 cells. (C) Cell viability determined by XTT assay. Cells were treated with PBS, Ad/gTRAIL, doxorubicin (ADR), ADR+Ad/GFP, and ADR+Ad/gTRAIL. The doses for adenovirus and doxorubicin were 1000 vp/cell and 0.5 μ M/ml, respectively. Cell viability is expressed relative to that of cells treated with PBS, which was set at 1. Values represent the means for quadruplicate wells (bars, \pm SD).

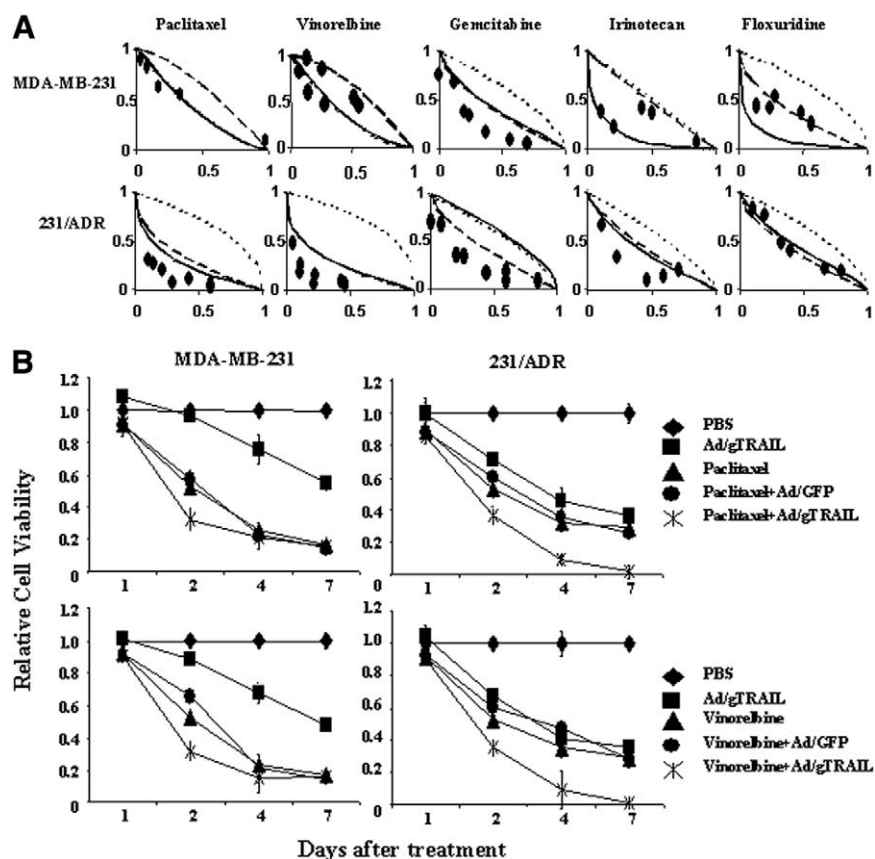
transgene expression in the lungs after aerosolized vector administration [36]. Therefore, the adenovector in a complex with protamine and hydrocortisone was used for gene delivery to the lung in this study.

We established lung metastatic tumor models in 6- to 8-week-old *nude* mice by the injection of 2×10^6 MDA-MB-231 or 231/ADR cells through the tail vein. Treatment was started 1 week after tumor cell inoculation. We evaluated the effect of Ad/gTRAIL plus doxorubicin in the MDA-MB-231 tumor model, whereas Ad/gTRAIL plus paclitaxel was evaluated in the 231/ADR tumor model. We used PBS and Ad/CMV-GFP as the mock control and vector control, respectively. Regimens for doxorubicin and paclitaxel were similar to those used clinically. The aerosolized adenovector was administered as described under Materials and Methods. Animals were euthanized 6 weeks after tumor cell inoculation, and metastatic tumors on the lung surface and in lung sections were evaluated. In

the mice bearing MDA-MB-231 tumors, treatment with Ad/gTRAIL or doxorubicin significantly decreased the number of tumors in the lung compared with the number in mock or Ad/CMV-GFP control groups ($P < 0.01$) (Figs. 3A and 3B). Moreover, the combination of Ad/gTRAIL and doxorubicin decreased the number of tumor nodules on the lung surface or in lung sections significantly more than either of the two agents alone.

Similar results were observed in mice bearing tumors derived from the doxorubicin-resistant breast cancer cell line 231/ADR. Specifically, lung tumor nodules were significantly suppressed in mice treated with Ad/gTRAIL or paclitaxel compared with those treated with Ad/CMV-GFP or PBS. Moreover, the suppression of lung metastasis was more dramatic in animals treated with Ad/gTRAIL plus paclitaxel than in the other animals. Specifically, lung tumor nodules in this group was significantly less than in any other groups ($P < 0.05$) (Fig. 4).

FIG. 2. Combination effect in doxorubicin-resistant cells. (A) IC_{50} isobolograms of the combined effects of Ad/gTRAIL and some chemotherapeutic agents in MDA-MB-231 and 231/ADR cell lines. The x and y axes represent equieffect doses for 50% cell killing by chemotherapeutic agent and Ad/gTRAIL, respectively. The observed data points are indicated by the filled symbols. (B) Cell viability determined by XTT assay. The m.o.i. of Ad/gTRAIL was 1000 vp/cell, and the concentrations of paclitaxel and vinorelbine were 1.5 and 1 nM, respectively. Values represent the means for quadruplicate wells (bars, \pm SD).



Minimal Toxicity of Ad/gTRAIL and Doxorubicin Combination Therapy

We also evaluated the toxicity of Ad/gTRAIL administered by aerosol in combination with systemic administration

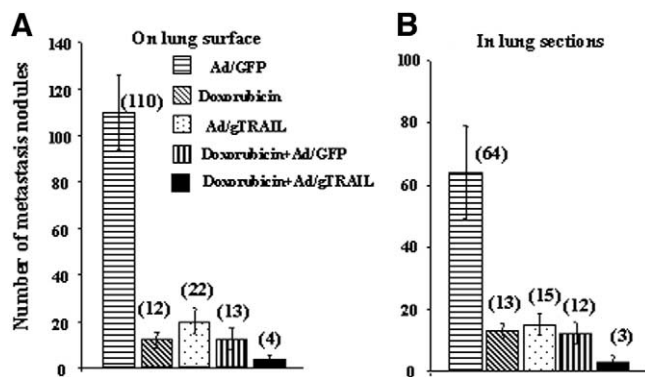


FIG. 3. Effects of Ad/gTRAIL and doxorubicin on lung metastases from breast cancer cell line MDA-MB-231. Mice were randomized into different groups. Ad/gTRAIL or Ad/GFP was administered by aerosol at 1×10^{12} particles/dose. Doxorubicin was administered at 4 mg/kg/dose by tail vein injection. Tumor nodules on the (A) lung surface and (B) lung sections were counted as described in the text. The values represent the means \pm SD of five mice.

of chemotherapeutic agents. For this purpose, we collected blood samples from the animals before the treatments, 1 week after the last treatment, and at the end of the experiment. We performed hemoglobin measurements, white blood cell and platelet counts, and serum liver enzyme assays of aspartate transaminase and alanine transaminase. All results were normal at all times. Further, no animals died during the treatment. These findings show that the *nude* mice tolerated this treatment.

We also compared the gene expression from Ad/hTERT-LacZ and Ad/CMV-LacZ in the lung metastasis model after aerosolized vector administration (Fig. 5A). We treated animals bearing lung tumors derived from MDA-MB-231 cells with Ad/hTERT-LacZ and Ad/CMV-LacZ administered by aerosol. We euthanized the animals 2 days after the treatments. Transgene expression from Ad/CMV-LacZ was observed in both cancerous and normal lung tissues. In contrast, X-gal blue staining occurred only in tumor tissue in animals treated with Ad/hTERT-LacZ. This showed that the hTERT promoter can be used to minimize transgene expression and associated toxicity in normal lung tissues. Adenovector DNA analysis of lung, heart, brain, liver, spleen, kidney, stomach, and intestine tissues collected 2 and 7 days after the aerosol-

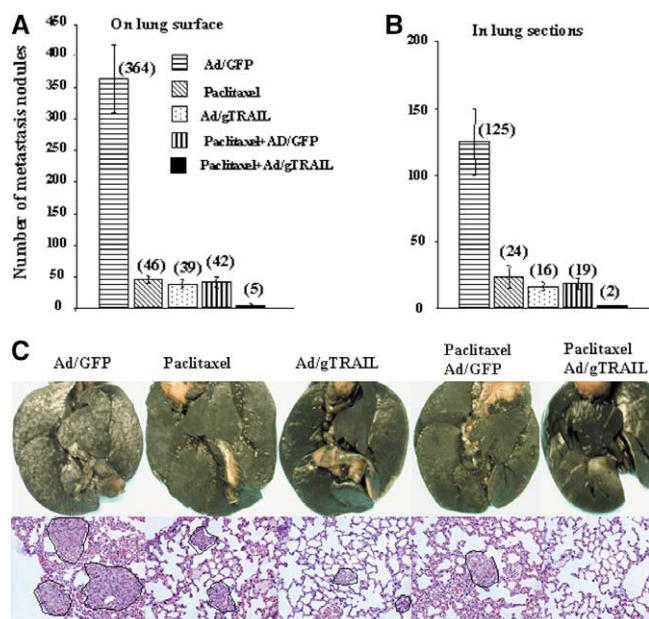


FIG. 4. Effects of Ad/gTRAIL plus paclitaxel in lung metastasis from the doxorubicin-resistant 231/ADR cell line. The experiment was performed as described for Fig. 3, except that paclitaxel (4 mg/kg) was used instead of doxorubicin. (A) The number of tumor nodules on lung surface and (B) the number of tumor nodules in lung sections. The values represent the means \pm SD of four mice. (C) Whole lung ink staining and H&E staining of lung sections. The treatment is indicated at the top.

ized administration showed a substantial amount of adenovector DNA in lung and stomach tissue but none in other organs, suggesting that aerosolized vectors were both inhaled and swallowed by mice (Fig. 5B). The viral genome was not detected in any of the organs tested 1 month after the aerosolized treatment.

DISCUSSION

We recently found that breast cancer cell lines that are resistant to recombinant TRAIL proteins are susceptible to an adenovector expressing the full-length TRAIL protein [12]. Others studying prostate cancer cell lines made a similar observation [14]. We also found that both doxorubicin-sensitive and doxorubicin-resistant breast cancer cell lines remained sensitive to treatment with TRAIL-expressing vectors. Here we have found that synergistic or additive effects can be achieved by a combination of TRAIL gene therapy and chemotherapy in doxorubicin-sensitive and doxorubicin-resistant breast cancer cell lines that are sensitive to treatment with TRAIL-expressing vectors. Because synergistic interactions can lead to a reduction in the dose of the component agents needed [15–17], any synergy that results from combined therapy is expected to improve the overall therapeutic results by reducing toxicity, enhancing efficacy, or both.

Synergistic effects of recombinant TRAIL with chemotherapeutic agents were also observed in other tumor models [18,19]. Lipkowitz and his associates [20] reported previously that almost all ovarian cancer cells that are resistant to chemotherapy are also resistant to recombinant TRAIL protein. The combination of TRAIL and chemotherapy overcomes this resistance in a synergistic fashion by triggering caspase-mediated apoptosis. Yet, no correlation between the induction of apoptosis and the level of death receptors was found. They also found that most breast cancer cell lines are resistant to the recombinant TRAIL protein [21]. Nevertheless, doxorubicin or 5-fluorouracil significantly augmented TRAIL-induced apoptosis in most breast cancer cell lines, suggesting that treatment with chemotherapy provides an approach to sensitizing breast cancer cells to TRAIL-induced apoptosis. Moreover, ionizing radiation [22], inhibition of casein kinase II [23], and inhibition of NF- κ B [24] have all been found to sensitize breast cancer cells to TRAIL-mediated apoptosis.

In this study, we also tested whether TRAIL gene therapy alone or in combination with chemotherapy can be used for the treatment of lung metastasis. Because all blood circulates through the lungs, this organ is one of the most common sites for metastasis. Indeed, autopsy

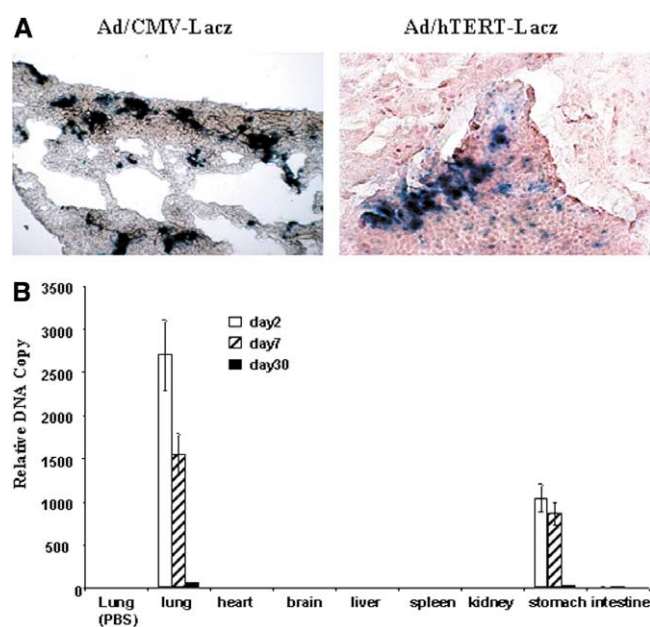


FIG. 5. Transgene expression after aerosolized administration. (A) LacZ expression after aerosolized administration of Ad/hTERT-LacZ and Ad/CMV-LacZ. Of note, treatment with Ad/CMV-LacZ resulted in lacZ expression in normal and tumor tissue, whereas treatment with Ad/hTERT-LacZ resulted in lacZ expression mainly in tumor tissue. (B) Vector distribution after aerosolized adenovector administration. Presence of viral genome was quantified by real-time PCR analysis of Ad/CMV-LacZ viral DNA in different organs after aerosolized administration. The values represent the means \pm SD of three mice/group.

studies have revealed that approximately one-third of cancer patients die with lung metastases and that some of them have metastases only in the lungs [25–27]. Therefore, it is desirable to develop strategies for the treatment of lung metastasis. To test the feasibility of gene therapy for lung metastasis, reliable and satisfactory metastasis models and gene delivery approaches are needed. Unfortunately, there is no animal tumor model that can be used as a satisfactory predictor of drug responsiveness of tumors in humans [28–30]. Theoretically, a murine model of spontaneous metastasis or a murine transgenic cancer model might be more predictive than transplanted tumors for the clinical outcome. In reality, however, spontaneous or transgenic murine tumors lack essential qualities (validity, reliability, and reproducibility) for most preclinical drug studies [31]. Time to tumor or metastasis development, organ sites, and numbers of tumor development are not predictable for most individual transgenic/knockout mice [32]. Moreover, each spontaneous tumor arising in the same birth-date batch of inbred mice, in the same organ, and induced by the same carcinogen is a separate and unique entity with its own unique histologic appearance, biological behavior, and drug-response characteristics [28]. Therefore, metastases derived from the intravenous injection of human cancer cell lines are still the most affordable, reproducible, and reliable models for preclinical studies.

Because the systemic administration of an adenovector results mainly in transduction of liver tissue, we used an aerosolized vector to administer the gene to the lung. The aerosolized administration of adenovectors for treatment of cystic fibrosis has been evaluated in clinical trials and has been shown to be feasible for distributing adenovectors throughout the conducting airways [33,34]. However, the adenovirus-mediated transduction of airway epithelial cells must be improved before gene therapy for cystic fibrosis will be effective [33,34]. More recently, it has also been reported that the transduction of lung tissue by adenovectors can be dramatically increased by using various formulations, including protamine [35]. We have therefore evaluated various adenovector formulations for aerosolized administration and found that a complex of the adenovector with protamine and hydrocortisone resulted in optimal gene expression in the lung [36]. However, the exact viral dose received per mouse and the immune responses to adenovectors delivered with this method are not yet characterized. Nevertheless, in this study, we found that aerosolized administration of Ad/gTRAIL in a complex with protamine and hydrocortisone significantly suppressed lung metastasis from both doxorubicin-sensitive and doxorubicin-resistant cancer cell lines in immunocompromised mice. Moreover, the metastasis suppression was more significant after the combination of TRAIL gene therapy and chemotherapy. This finding suggests that a combination of TRAIL gene therapy and chemotherapy may have clinical applications.

MATERIALS AND METHODS

Cell lines and reagents. Human breast cancer cell lines MCF7, MDA-MB-231, MDA-MB-453, and MDA-MB-468 were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, antibiotics, and glutamine. Doxorubicin-resistant 231/ADR cells were obtained through the stepwise exposure of the parental cells to doxorubicin as described previously [12]. The chemotherapeutic agents used in this study were obtained from the M. D. Anderson Cancer Center Pharmacy. They were paclitaxel (Bristol-Myers Squibb Co., Princeton, NJ), gemcitabine (Eli Lilly and Co., Indianapolis, IN), vinorelbine (Pierre Fabre, Idron, France), irinotecan (Pharmacia & Upjohn Co., Kalamazoo, MI), floxuridine (Ben Venue Labs, Inc., Bedford, OH), protamine sulfate (American Pharmaceutical Partners, Inc., Los Angeles, CA), and hydrocortisone (Sigma Chemical Co., St. Louis, MO). Each agent was further diluted to proper concentrations with phosphate-buffered saline.

Adenovectors. Adenovectors Ad/CMV-LacZ, Ad/hTERT-LacZ, Ad/CMV-GFP, and Ad/gTRAIL have been described previously [11,37]. The expansion, purification, titration, and quality analyses of all vectors used were performed at the vector core facility of our institution as described previously [11,37]. All viral preparations were shown by polymerase chain reaction (PCR) to be free of the E1⁺ adenovirus [37] and by use of a *Limulus* ameocyte lysate endotoxin detection kit (BioWhittaker, Walkersville, MD) to be free of endotoxin. The titer used in this study was determined by the absorbency of the dissociated virus at A_{260nm} (one A_{260nm} unit = 10¹² viral particles/ml), and titers determined using a plaque assay were used to determine additive information. Particle:infectious unit (IU) ratios were usually between 30:1 and 100:1. Thus, the m.o.i. of 1000 vp is equivalent to an m.o.i. of 10–30 IU. The m.o.i. for each of the cell lines was 1000 particles/cell. Unless otherwise specified, Ad/CMV-GFP was used as a vector control and PBS as a mock control.

Cell viability assay and isobologram analysis. Cell viability was determined by an XTT assay (Cell Proliferation Kit II; Roche Molecular Biochemicals, Indianapolis, IN) as described previously [11,37]. Briefly, 1 day after the cells were cultured in 96-well plates, different m.o.i. (from 125 to 8000) of adenovirus and/or varying concentrations of chemotherapeutic agents (from 0.3 nM to 50 μM) were added to each well. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Cell growth and viability were quantified using XTT assay on day 4 after treatment. Each experiment was performed in quadruplicate and repeated at least twice. The effects of the combination of Ad/gTRAIL and other cytotoxic agents *in vitro* were analyzed using two-dimensional isobolograms as described previously [13] (for detailed information, see [38]). Detailed mathematic methods for isobologram analysis will be available upon request. The effects of the combined therapeutic agents on tumor cell growth were analyzed quantitatively and statistically by plotting the observed experimental data onto the corresponding isobolograms. The combinations with data points falling mainly below the envelope, within the envelope, or above the envelope were deemed to have synergism, additive effects, or antagonism, respectively.

Biochemical analysis. LacZ gene expression was analyzed as described previously [37,39]. Briefly, cells or mouse tissues were homogenized in a β-galactosidase assay buffer. Tissue debris was removed by microcentrifugation. The protein concentrations in the supernatant were measured using a kit (Pierce Chemical Company, Rockford, IL). β-Galactosidase activities were determined using a luminometer and a Galacto Light chemiluminescence assay kit (Tropix, Inc., Bedford, MA). For 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) staining, 8-μm frozen sections were fixed with 0.5% glutaraldehyde for 15 min at 4°C before being stained with a solution containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 15 mM NaCl, 44 mM HEPES (pH 7.3), and 0.5 mg/ml X-gal at 37°C overnight. The next day, sections were counterstained with nuclear fast red (Sigma Chemical Co.). The flow cytometry assay was performed as described previously [11,37,40]. In brief, both adherent and floating cells were harvested 96 h after treatment. One part was used for the analysis of GFP expression, which involved using a FACS system (Becton-Dickinson,

Mansfield, MA) to determine the percentage of cells that were GFP-positive. The second part of the sample, which was fixed by 70% ethanol overnight and stained with propidium iodide (1 ml PI (50 μ g/ml), 10 μ l RNase, 9 ml PBS) before analysis, was used to quantify the apoptotic cells. This was done using flow cytometry to measure the sub-G0/G1 cellular DNA content using Cell Quest software (Becton–Dickinson, San Jose, CA) [40].

Animal experiments. Animal experiments were carried out in accordance with the *Guidelines for the Care and Use of Laboratory Animals* (NIH Publication No. 85-23) and the institutional guidelines of The University of Texas M. D. Anderson Cancer Center. Human breast cancer lung metastatic tumor models were established in 6- to 8-week-old *nude* mice (Charles River Laboratories, Inc., Wilmington, MA) by injecting 2×10^6 MDA-MB-231 or 231/ADR breast cancer cells/0.2 ml into the tail vein of each mouse on day 1. Animals were then randomized into different treatment groups. Treatment was started 1 week after the cells were inoculated into the mice. Doxorubicin or paclitaxel was administered intravenously via the tail vein on days 7 and 28 (4 mg/kg). Adenovirus (Ad/GFP or Ad/gTRAIL) was administered by aerosol on days 7, 14, 21, 28, and 35. Unless otherwise described, all aerosolized administrations of adenovectors were performed as the following: 1×10^{12} viral particles were diluted in PBS to a final volume of 1 ml and mixed with 500 μ l of PBS containing 5 mg of protamine. The protamine–adenovirus complex was then mixed with 1.25 mg of hydrocortisone in PBS to a final volume of 5 ml. After incubating 10 min at room temperature, the complex was placed into a nebulizer chamber. The aerosol from the Inspiration 626 nebulizer (Respironics Healthscan, Inc., Cedar Grove, NJ) was passed through a sealed plastic cage ($20 \times 20 \times 30$ cm) that housed up to 10 mice. The exposure lasted for approximately 60 min.

Ten mice from each group were euthanized on day 42 after tumor cell inoculation. Lung, heart, brain, liver, spleen, kidney, stomach, and intestine tissues were harvested for pathologic study. For calculating metastasis nodules on the lung surface, 5 mice from each group were injected with 15% India ink (85 ml dH₂O, 4 drops of ammonium hydroxide, and 15 ml Higgins India ink) through the trachea and were stained in Feketes solution (700 ml ethanol, 300 ml dH₂O, 100 ml formaldehyde, and 50 ml acetic acid). White nodules on the lung surface were counted under a surgical microscope. Metastasis nodules on lung sections were counted in 4 or 5 mice from each group, three sections per mouse. Histopathologic analysis was performed in the Histology Laboratory of the Department of Veterinary Medicine and Surgery at M. D. Anderson Cancer Center. For biodistribution of adenovirus after aerosolized vector administration, 5 animals from each group were euthanized and lung, heart, brain, liver, spleen, stomach, and intestine tissues were harvested on days 2, 7, and 30 after treatment with Ad/CMV-LacZ or Ad/hTERT-LacZ. LacZ gene expression was determined as described above. The presence of the viral genome in various organs was analyzed using PCR as previously described.

Statistical analysis. Differences among the treatment groups were assessed by ANOVA using statistical software (Statistica, Tulsa, OK). A value of $P \leq 0.05$ was considered significant. The drug concentration that inhibited cell growth by 50% (IC₅₀) was calculated by Curve Expert 1.3 software (Starkville, MS).

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