Chemoinducible gene therapy: A strategy to enhance doxorubicin antitumor activity

Carlos A. Lopez,[1] Eric T. Kimchi,[1] Helena J. Mauceri,[2] James O. Park,[1] Neil Mehta,[2] Kevin T. Murphy,[2] Michael A. Beckett,[2] Samuel Hellman,[2] Mitchell C. Posner,[1] Donald W. Kufe,[3] and Ralph R. Weichselbaum[2]

Departments of [1]Surgery and [2]Radiation and Cellular Oncology, University of Chicago, Chicago, Illinois and [3]Dana-Farber Cancer Institute, Boston, Massachusetts

Abstract

A replication-defective adenoviral vector, Ad.Egr-TNF.11D, was engineered by ligating the CArG ($CC(A/T)_6GG$) elements of the Egr-1 gene promoter upstream to a cDNA encoding human tumor necrosis factor-α. We report here that Ad.Egr-TNF.11D is activated by the clinically important anticancer agents cisplatin, cyclophosphamide, doxorubicin, 5-fluorouracil, gemcitabine, and paclitaxel. *N*-acetylcysteine, a free radical scavenger, blocked induction of tumor necrosis factor-α by anticancer agents, supporting a role for reactive oxygen intermediates in activation of the CArG sequences. Importantly, resistance of PC-3 human prostate carcinoma and PROb rat colon carcinoma tumors to doxorubicin *in vivo* was reversed by combining doxorubicin with Ad.Egr-TNF and resulted in significant antitumor effects. Treatment with Ad.Egr-TNF.11D has been associated with inhibition of tumor angiogenesis. In this context, a significant decrease in tumor microvessel density was observed following combined treatment with doxorubicin and Ad.Egr-TNF.11D as compared with either agent alone. These data show that Ad.Egr-TNF.11D is activated by diverse anticancer drugs. [Mol Cancer Ther 2004;3(9):1167–75]

Introduction

Tumor necrosis factor-α (TNF-α) is a cytokine produced by a variety of cells including macrophages, lymphocytes, and natural killer cells. TNF-α is directly cytotoxic to some tumor cells *in vitro*, although direct cell killing frequently requires inhibition of protein synthesis with compounds such as cycloheximide (1–3). The antitumor activity of TNF-α is predominantly mediated by destruction of the tumor vasculature (4) and this cytokine was named for its induction of hemorrhagic necrosis in experimental tumors (5, 6). Based on antitumor effects in animal models, clinical trials were done using i.v. delivery of TNF-α. The therapeutic utility of TNF-α, however, was limited by serious side effects, which included fatigue, weight loss, nausea, cachexia, and shock (7–12). To decrease systemic toxicity of TNF-α, regional delivery approaches were developed to restrict TNF-α to the tumor bed in isolated limb and liver perfusions (13–18). These strategies have shown promise in some clinical settings but require surgical intervention and their own associated toxicities.

The clinical effectiveness of cancer gene therapy has been limited by (*a*) lack of control of therapeutic gene expression within the tumor and (*b*) selective targeting of the vector to the tumor. Several strategies have been proposed for the control of gene expression. One strategy is transcriptional targeting in which the promoter regulating the therapeutic gene is activated by tumor-selective transcription factors. Examples include the use of the MUC-1 promoter in breast cancer and the carcinoembryonic antigen promoter in colon cancer (19, 20). In a transcriptional targeting strategy to localize TNF-α induction to the tumor bed, ionizing radiation was employed to activate the radioinducible CArG ($CC(A/T)_6GG$) sequences of the Egr-1 promoter ligated upstream of a cDNA encoding the human TNF-α gene. For delivery, the Egr-TNF construct was integrated into a replication-defective adenovirus (E1, partially E3 deleted) to construct the Ad.Egr-TNF vector (21). Preclinical experiments showed synergistic antitumor effects following combined treatment with Ad.Egr-TNF and ionizing radiation in human head and neck, prostate, esophageal, and glioma xenografts (21–25). TNF-α production was confined to the tumor bed and no systemic toxicity was detected. Histopathologic analyses showed damage to the tumor microvasculature but not to the adjacent normal tissues (26).

Ad.Egr-TNF.11D has been studied in two separate phase I clinical trials with radiation therapy (27–30). The first trial included patients with tumors of different histologic types who required palliative radiotherapy. Tumors were directly injected with the Ad.Egr-TNF.11D vector at concentrations of vector ranging from 4×10^7 to 4×10^{11} particle units. The doses of radiation ranged from 30 to 66.6 Gy. Seventy percent (21 of 30) of the patients showed a tumor response or tumor stabilization, which was noted

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Requests for reprints: Ralph R. Weichselbaum, Department of Radiation and Cellular Oncology, University of Chicago Hospitals, Center for Advanced Medicine, Room 1329, Mail Code 9006, 5758 South Maryland Avenue, Chicago, IL 60637. Phone: 773-702-0817; Fax: 773-834-7233. E-mail: rrw@rover.uchicago.edu

mostly at the higher dose levels (4×10^9–4×10^{11} particle units) of the vector. There were five complete responses, which included three patients with melanoma, a typically radioresistant histologic tumor, and one patient with rectal cancer and another with breast cancer (31). In the second phase I trial, patients with large unresectable soft tissue sarcomas of the extremities were treated with Ad.Egr-TNF.11D (4×10^9–4×10^{11} particle units in 1 log increments) and 50 Gy. Objective responses were observed in 11 of 13 (85%) patients. Pathologic complete responses were noted in two patients with very large tumors (328–338 cm^2). Eight patients exhibited a partial response. Four patients experienced 95% tumor necrosis, three patients experienced 80% necrosis, and one patient experienced 60% necrosis.[4] Taken together, these findings show the safety of Ad.Egr-TNF.11D plus ionizing radiation combined treatment (28, 30). Additionally, sterilization of radioresistant and/or very large tumors suggests that Ad.Egr-TNF.11D may enhance radiocurability in some patients.

A common feature of anticancer agents is the production of oxygen and other free radical species that lead to DNA damage, peroxidation of lipids, protein modification, and cellular death (32, 33). Agents other than ionizing radiation that increase intracellular reactive oxygen intermediate (ROI; ref. 34) include the widely used anticancer drugs doxorubicin (35), cisplatin (36, 37), cyclophosphamide (38), 5-fluorouracil (5-FU; ref. 39), gemcitabine (40), and paclitaxel (41). Previous studies showed that ionizing radiation activates the transcription of the Egr-1 CArG sequences by production of ROIs (42, 43). We therefore hypothesized that clinically employed chemotherapeutic agents that increase ROIs could also be employed to activate Ad.Egr-TNF.11D in a chemoinducible gene therapy strategy.

We report that Ad.Egr-TNF.11D can be activated by anthracyclines, alkylating agents, antimetabolites, and microtubule-stabilizing agents through the production of ROIs. Importantly, combined treatment with doxorubicin and Ad.Egr-TNF.11D produces greater antitumor effects than either agent alone in tumor models that are resistant to doxorubicin. These antitumor effects were achieved by selective induction of Ad.Egr-TNF.11D within the tumor volume, inhibition of tumor angiogenesis, and/or direct cytotoxic effects mediated by the combination of Ad.Egr-TNF.11D and doxorubicin. Taken together, these data support chemoinducible gene therapy to overcome tumor resistance to broad classes of cancer chemotherapeutic agents.

Materials and Methods

Cell Culture

The human prostate carcinoma cell line PC-3 (American Type Culture Collection, Manassas, VA) was maintained in DMEM-F12 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with fetal bovine serum (10% v/v, Intergen, Purchase, NY), penicillin (100 IU/mL), and streptomycin (100 μg/mL, Invitrogen Life Technologies) at 37°C with 7.5% CO_2. PC-3 cells are p53 null (44) and express P-glycoprotein, multidrug resistance–associated protein, glutathione *S*-transferase π (45), and Bcl-2 (46). The rat colon adenocarcinoma cell line DHD/K12/TRb(PROb) was obtained from Francois Martin (University of Dijon, Dijon, France) and was established in BD-IX rats by injection of 1,2-dimethylhydrazine. PROb cells were maintained in DMEM (Invitrogen Life Technologies) supplemented with fetal bovine serum (10% v/v, Intergen), penicillin (100 IU/mL), and streptomycin (100 μg/mL, Invitrogen Life Technologies) at 37°C with 7.5% CO_2. There is little published information on the molecular/genetic characteristics of PROb cells.

Viral Vector

Ad.Egr.TNF.11D (GenVec, Inc., Gaithersburg, MD), a replication-deficient adenoviral vector (E1, partially E3 and E4 deleted) containing the human TNF-α gene under the control of the radiation-inducible promoter Egr-1, was stored at −80°C and was diluted in formulation buffer (GenVec) to the appropriate concentration.

Chemical Reagents

N-acetylcysteine was obtained from Roxane Laboratories, Inc. (Columbus, OH). Cisplatin and 5-FU were obtained from American Pharmaceutical Partners (Schaumburg, IL). Doxorubicin was manufactured by Ben Venue Laboratories (Bedford, OH). Gemcitabine was obtained from Eli Lilly (Indianapolis, IN). Paclitaxel was manufactured by F.H. Faulding (Mulgrave, Victoria, Australia). Cyclophosphamide was obtained from Bristol-Myers Squibb (Princeton, NJ).

Xenografts

PC-3 xenografts were established by injection of 10^7 cells in 100 μL PBS into the right hind limb of 6-week-old female athymic nude mice (Frederick Cancer Research Institute, Frederick, MD). PROb xenografts were established by injecting 5×10^6 cells in 100 μL PBS. Experiments were conducted 2 to 3 weeks after injection when tumors reached an average size of 200 to 300 mm^3. Experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the University of Chicago.

Chemosensitivity of PC-3 and PROb Cells as Determined by 3-(4,5-Dimethylthiazol-2-yl)-5-(3-Carboxymethoxyphenyl)-2-(4-Sulfophenyl)-2H-Tetrazolium, Inner Salt Assay

PC-3 and PROb cells were plated at a density of 10^5 cells in 100 μL medium per well in flat-bottomed 96-well tissue culture plates and incubated overnight. The medium was removed and cells were infected with Ad.Egr.TNF.11D in serum-free medium at 0 and 100 multiplicities of infection for 3 hours. After incubation, 200 μL complete medium with or without chemotherapeutic agents was added. Chemotherapeutic agents used were cisplatin at final concentrations of 46 and 460 μmol/L, doxorubicin at 3 and 300 μmol/L, 5-FU at 2 and 200 mmol/L, and paclitaxel at 1.4 and 140 μmol/L. Medium was removed 24 hours later and

[4] Mundt et al., Clinical Cancer Research. In press.

each well was rinsed with 200 μL complete medium and aspirated. Complete medium (100 μL) was added with 20 μL CellTiter 96 AQueous One Solution Cell Proliferation Assay solution [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay, Promega, Madison, WI]. Cells were allowed to incubate for 1 hour. Absorbance was measured at 490 to 650 nm.

Chemoinducibility of Ad.Egr-TNF.11D *In vitro*

PC-3 and PROb cells were plated at a density of 10^5 cells in 100 μL complete medium per well in flat-bottomed 96-well tissue culture plates and incubated overnight. The medium was removed and cells were infected with Ad.Egr.TNF.11D (GenVec) at 0 and 100 multiplicities of infection in 100 μL serum-free medium for 3 hours. After incubation, 200 μL complete medium with or without chemotherapeutic agents was added. The chemotherapeutic agents used were cisplatin at a final concentration of 250 μmol/L, doxorubicin at 3 μmol/L, 5-FU at 100 mmol/L, gemcitabine at 3 mmol/L, and paclitaxel at 14 μmol/L. Conditioned medium was harvested 24 hours later and TNF-α concentration was measured using a Quantikine Human TNF-α ELISA kit (R&D Systems, Minneapolis, MN).

Chemoinducibility of Ad.Egr-TNF.11D *In vivo*

PC-3 and PROb xenografts were injected i.t. with 5×10^9 particle units of Ad.Egr.TNF.11D on days 0 and 1. Chemotherapeutic agents given i.p. on days 1 and 2 included cisplatin (9 mg/kg), cyclophosphamide (160 mg/kg), doxorubicin (15 mg/kg), 5-FU (100 mg/kg), and gemcitabine (500 mg/kg). The control group received normal saline. Animals were euthanized and xenografts were harvested 24 hours after the second i.p. injection. Xenografts were snap frozen in liquid nitrogen and homogenized in radioimmunoprecipitation assay buffer [150 mmol/L NaCl, 10 mmol/L Tris (pH 7.5), 5 mmol/L EDTA (pH 7.5), 100 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 2 μg/mL aprotinin] using a Brinkman Polytron homogenizer (Kinematica AG, Lucerne, Switzerland). After three freeze-thaw lysis cycles, the homogenate was centrifuged at 7,800 × *g* in a Sorvall RC-5C SS34 rotor (Kendro Laboratory Products, Newtown, CT) for 10 minutes at 4°C. TNF-α levels in the supernatants were measured by ELISA as described above.

N-Acetylcysteine Effects on TNF-α Production *In vitro*

PC-3 and PROb cells were plated and infected with Ad.Egr-TNF.11D as described above. PC-3 and PROb cells were treated with *N*-acetylcysteine at 0, 10, 20, and 30 mmol/L followed immediately by the addition of 100 mmol/L 5-FU. Conditioned medium was collected after 24 hours of incubation at 37°C and stored at −20°C. TNF-α levels were determined by ELISA.

PC-3 and PROb cells were plated and infected with Ad.Egr-TNF.11D as described above. Prior to the addition of chemotherapeutic agents (cisplatin, doxorubicin, 5-FU, gemcitabine, and paclitaxel), 20 mmol/L *N*-acetylcysteine in 0.1 mL complete medium was added to each well. Conditioned medium was collected after 24 hours of incubation at 37°C and stored at −20°C. TNF-α levels were determined by ELISA.

Xenograft Regrowth Studies

PC-3 and PROb xenografts were established in nude mice as described (23, 47). Treatment was initiated on day 0 at which time mice were assigned to one of four treatment groups: control, doxorubicin, Ad.Egr-TNF.11D, and combination of Ad.Egr-TNF.11D and doxorubicin. On days 0 and 3, mice received i.t. injection of 10 μL of either 5×10^9 particle units Ad.Egr-TNF.11D (vector alone and combination groups) or 10 μL viral formulation buffer (control and doxorubicin groups). I.p. injections of doxorubicin (2 mg/kg) or an equal volume of normal saline were given daily from days 0 to 8. Xenografts were measured twice weekly and tumor volume was calculated according to the following formula: (length × width × thickness)/2 (21). Fractional tumor volumes (V/V_0, where V_0 is volume on day 0) were calculated and plotted.

Analysis of Microvessel Density

Two or three xenografts from each treatment group in the PC-3 regrowth study above, including control, doxorubicin alone, Ad.Egr-TNF.11D alone, and combination of Ad.Egr-TNF.11D plus doxorubicin, were collected and fixed in 10% neutral buffered formalin. Tissues were embedded in paraffin, cut in 5 μm slices, mounted, baked, cleared in xylene, and rehydrated in decreasing alcohol concentrations (100% to 70%) and distilled water. Sections were microwaved in 10 mmol/L citrate buffer (pH 6.0) for 18 minutes, washed, and soaked in 1% H_2O_2/methanol for 20 minutes prior to blocking with avidin-biotin (Vector Laboratories, Burlingame, CA) for 15 minutes. Slides were incubated with biotin (15 minutes), washed, and blocked with serum-free DAKO protein (DAKO, Carpinteria, CA) for 10 minutes prior to incubation with a 1:50 dilution of goat anti-mouse CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 60 minutes at room temperature. CD31 staining was visualized on tissue sections following incubation with DAKO biotinylated anti-goat secondary antibody for 30 minutes and 3,3′-diaminobenzidine reagent (Vector Laboratories) for 60 seconds. Sections were counterstained with Gill 3 hematoxylin and dehydrated in ethanol (95–100%) and xylene prior to mounting. All slides were read by an investigator blinded to the treatment groups. Positively stained vessels were counted in 5 to 10 high-power fields (×400) per slide using light microscopy. Blood vessels were identified by endothelial cell staining and by endothelial cells surrounding intraluminal erythrocytes.

Statistical Analysis

Statistical significance was determined by one-way ANOVA. Differences between treatment groups were determined by either Student's *t* test or Mann-Whitney rank sum test.

Results

Chemosensitivity of PC-3 and PROb Cells

Following infection with Ad.Egr-TNF.11D (100 multiplicities of infection), percentage survival in the presence of chemotherapy was compared with survival in complete medium without chemotherapy. PC-3 cells showed

surviving fractions of 60% (460 μmol/L) and 90% (46 μmol/L) with cisplatin, 30% (300 μmol/L) and 90% (3 μmol/L) with doxorubicin, 20% (200 mmol/L) and 80% (2 mmol/L) with 5-FU, and 10% (140 μmol/L) and 80% (1.4 μmol/L) with taxol. PROb showed surviving fractions of 77% (460 μmol/L) and 77% (46 μmol/L) with cisplatin, 85% (300 μmol/L) and 100% (3 μmol/L) with doxorubicin, 38% (200 mmol/L) and 69% (2 mmol/L) with 5-FU, and 8% (140 μmol/L) and 85% (1.4 μmol/L) with taxol.

Different Classes of Chemotherapeutic Agents Induce TNF-α Production by PC-3 and PROb Tumor Cells Infected with Ad.Egr-TNF.11D

Using an ELISA specific for human TNF-α, TNF-α production was assessed following infection of PC-3 and PROb cells with 100 multiplicities of infection of Ad.Egr-TNF.11D. Neither of these cell lines produced endogenous human TNF-α. Following infection with Ad.Egr-TNF.11D, PC-3 cells produce 14 pg/mL TNF-α and PROb cells produce 130 pg/mL. Next, PC-3 and PROb cells were infected with Ad.Egr-TNF.11D and exposed to cisplatin (250 μmol/L), doxorubicin (3 μmol/L), 5-FU (100 mmol/L), gemcitabine (3 mmol/L), or paclitaxel (14 μmol/L) based on LD_{50} values on a panel of human tumor cell lines.[5] Induction of TNF-α by cyclophosphamide was not investigated *in vitro* because this drug requires hepatic activation. In PC-3 cells infected with Ad.Egr-TNF.11D, significant increases in TNF-α levels were detected following exposure to cisplatin (3.8-fold increase), 5-FU (67.4-fold increase), gemcitabine (2.7-fold increase), and paclitaxel (1.7-fold increase; $P < 0.001$; Fig. 1A). Induction of TNF-α by doxorubicin was not evaluated because doxorubicin was toxic to PC-3 cells at the doses used in these experiments. Similar results were obtained using PROb cells infected with Ad.Egr-TNF.11D. Significant increases in TNF-α levels were found following exposure to cisplatin (1.3-fold increase; $P = 0.04$), 5-FU (1.7-fold increase; $P < 0.02$), gemcitabine (3.5-fold increase; $P < 0.001$), and paclitaxel (4.5-fold increase; $P < 0.001$; Fig. 1B). The greatest induction of TNF-α in PROb cells was observed following infection with Ad.Egr-TNF.11D and exposure to doxorubicin (7.4-fold increase; $P < 0.001$). These data obtained from histologically different cancer cell lines show that Ad.Egr-TNF.11D is activated by different classes of chemotherapeutic agents.

N-Acetylcysteine Alters Induction of Ad.Egr-TNF.11D by Chemotherapy

Based on previous studies that showed transcriptional activation of the Egr-1 promoter through the CArG sequences by ionizing radiation–mediated ROIs, we hypothesized that chemotherapeutic agents reported to induce intracellular ROIs would also activate Ad.Egr-TNF.11D and produce therapeutic levels of TNF-α protein. Notably, cisplatin (36, 37), cyclophosphamide (38), doxorubicin, 5-FU (39), gemcitabine (40), and paclitaxel (41) have been reported to induce intracellular ROIs and/or intracellular changes in redox potential. Consequently, we tested whether *N*-acetylcysteine, a free radical scavenger, would decrease TNF-α production if present at the time of addition of chemotherapeutic agents.

First, we examined the effect of *N*-acetylcysteine on TNF-α production following exposure to 5-FU. Figure 2A shows that increasing concentrations of *N*-acetylcysteine (10–30 mmol/L) decrease the concentration of TNF-α protein produced by PC-3 cells infected with Ad.Egr-TNF.11D and treated with 100 mmol/L 5-FU compared with PC-3 cells infected with Ad.Egr-TNF.11D alone. We next investigated the effect of *N*-acetylcysteine on TNF-α induction by the same panel of chemotherapeutic agents used in the *in vitro* chemoinduction experiments. *N*-acetylcysteine significantly decreased the concentration of TNF-α protein produced by Ad.Egr-TNF.11D transduced PC-3 cells

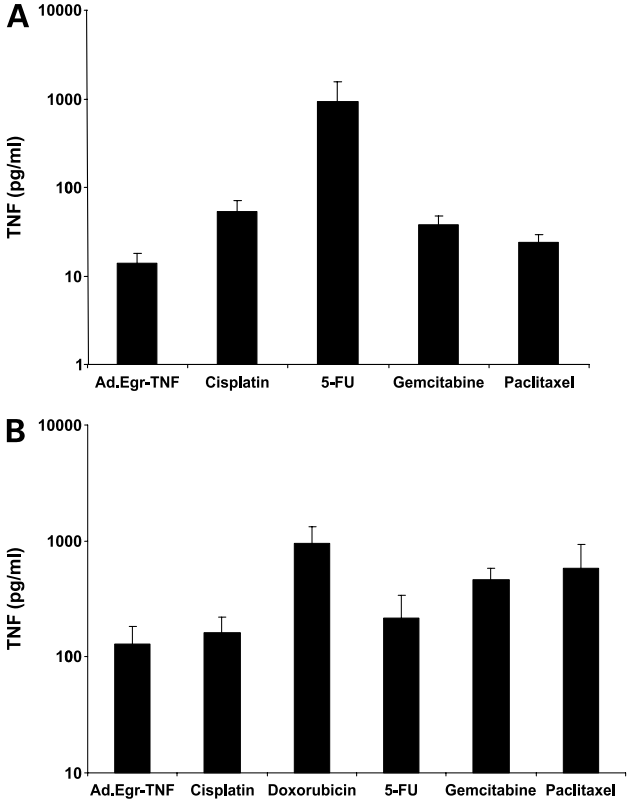

Figure 1. Induction of TNF-α protein. TNF-α production by Ad.Egr-TNF.11D-infected cells exposed to cisplatin (250 μmol/L), doxorubicin (3 μmol/L), 5-FU (100 mmol/L), gemcitabine (3 mmol/L), or paclitaxel (14 μmol/L) for 24 hours was measured by ELISA. **A,** significant increases in levels of TNF-α protein were detected in PC-3 cells following exposure to Ad.Egr-TNF.11D + cisplatin (53.2 pg/mL; $P < 0.001$), 5-FU (943.7 pg/mL; $P < 0.001$), gemcitabine (38.3 pg/mL; $P < 0.001$), and paclitaxel (23.8 pg/mL; $P < 0.001$) compared with exposure to Ad.Egr-TNF.11D alone (14 pg/mL). Doxorubicin was toxic to PC-3 cells. **B,** PROb cells infected with Ad.Egr-TNF.11D produced 130 pg/mL TNF-α protein. The combination of Ad.Egr-TNF.11D and chemotherapeutic agents significantly increased TNF-α levels: cisplatin (163.3 pg/mL; $P < 0.04$), doxorubicin (961.9 pg/mL; $P < 0.001$), 5-FU (215.9 pg/mL; $P = 0.02$), gemcitabine (460 pg/mL; $P < 0.001$), and paclitaxel (583.2 pg/mL; $P < 0.001$).

[5] See http://dtp.nci.nih.gov/branches/btb/ivclsp.html.

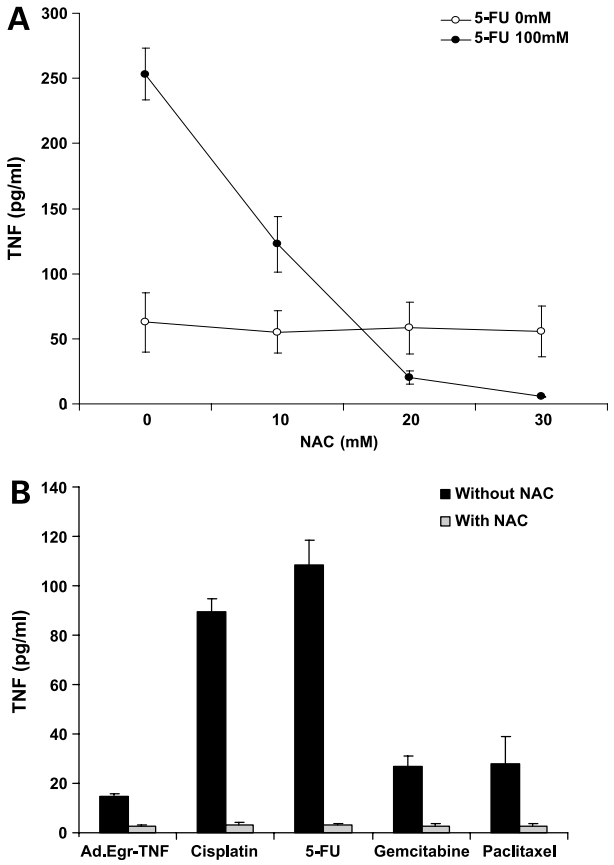

Figure 2. The effect of *N*-acetylcysteine (*NAC*) on the induction of TNF-α protein by PC-3 cells. **A,** in the presence of increasing concentrations of *N*-acetylcysteine (10 – 30 mmol/L), TNF-α production by PC-3 cells infected with Ad.Egr-TNF.11D and treated with 5-FU (0 and 100 mmol/L) falls below constitutive levels from PC-3 cells infected with Ad.Egr-TNF.11D alone. *Points,* mean; *bars,* SEM. **B,** effect of *N*-acetylcysteine on the chemoinduction of TNF-α protein TNF-α production by Ad.Egr-TNF.11D-infected cells exposed to cisplatin (250 μmol/L), doxorubicin (3 μmol/L), 5-FU (100 mmol/L), gemcitabine (3 mmol/L), or paclitaxel (14 μmol/L) with or without addition of *N*-acetylcysteine (200 mmol/L) was measured by ELISA. In PC-3 cells (**A**) and PROb cells (**B**), the addition of *N*-acetylcysteine significantly reduced the TNF-α levels induced by the panel of chemotherapeutic agents tested.

treated with cisplatin, 5-FU, gemcitabine, and paclitaxel ($P \leq 0.042$; Fig. 2B). The induction of TNF-α following treatment with 3 μmol/L doxorubicin in PROb cells was significantly reduced ($P < 0.001$) in the presence of *N*-acetylcysteine. Similar results were obtained when PROb cells were treated with cisplatin, 5-FU, gemcitabine, or paclitaxel and exposed to *N*-acetylcysteine (data not shown).

In vivo Induction of TNF-α in PC-3 and PROb Xenografts following Injection with Ad.Egr-TNF.11D and Treatment with Chemotherapeutic Agents

We next investigated the induction of human TNF-α by chemotherapeutic agents in PC-3 and PROb tumors growing in nude mice. Xenografts were injected with Ad.Egr-TNF.11D on days 0 and 1 and chemotherapy was given on days 1 and 2. Significant increases in human TNF-α levels in the tumors were detected 48 hours after the second injection of Ad.Egr-TNF.11D. PC-3 tumors injected with Ad.Egr-TNF.11D alone produced 376.33 ± 64.22 pg/mg of TNF-α protein. The combination of Ad.Egr-TNF.11D and chemotherapy produced a significant increase in TNF-α levels following treatment with cisplatin (3.1-fold increase; $P = 0.062$), cyclophosphamide (4.4-fold increase; $P < 0.001$), doxorubicin (4.2-fold increase; $P < 0.001$), 5-FU (4.4-fold increase; $P < 0.001$), and gemcitabine (3.1-fold increase; $P < 0.001$; Fig. 3A). In PROb xenografts, significant induction of TNF-α protein was detected following combined treatment with Ad.Egr-TNF.11D and cisplatin (2.6-fold increase; $P = 0.002$), cyclophosphamide (3.0-fold increase; $P < 0.001$), doxorubicin (2.3-fold increase; $P < 0.001$), 5-FU (1.9-fold increase; $P = 0.023$), and gemcitabine (2.5-fold increase; $P < 0.001$) compared with treatment with Ad.Egr-TNF.11D alone (Fig. 3B). Studies of *in vivo*

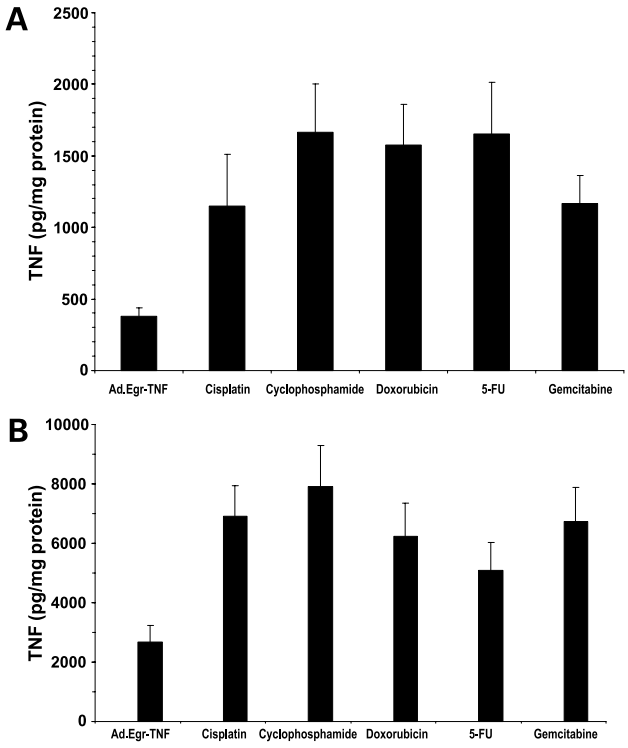

Figure 3. *In vivo* measurement of TNF-α protein. **A,** PC-3 xenografts. A significant increase in TNF-α protein concentration was observed following treatment with Ad.Egr-TNF.11D and cisplatin (1,150.91 ± 361.35 pg/mg protein; $P = 0.062$), cyclophosphamide (1,661.83 ± 343.12 pg/mg protein; $P < 0.001$), doxorubicin (1,577.27 ± 284.35 pg/mg protein; $P < 0.001$), 5-FU (1,653.33 ± 362.70 pg/mg protein; $P < 0.001$), and gemcitabine (1,169.09 ± 195.47 pg/mg protein; $P < 0.001$) compared with Ad.Egr-TNF.11D treatment alone (376.33 ± 64.22 pg/mg protein). **B,** significant induction of TNF-α was also detected in PROb tumors following combined treatment with Ad.Egr-TNF.11D and chemotherapy including cisplatin (6,912.50 ± 1,013.73 pg/mg protein; $P = 0.002$), cyclophosphamide (7,923.53 ± 1,362.56 pg/mg protein; $P < 0.001$), doxorubicin (6,229.41 ± 1,137.10 pg/mg protein; $P < 0.001$), 5-FU (5,094.12 ± 923.81 pg/mg protein; $P = 0.023$), and gemcitabine (6,723.53 ± 1,173.06 pg/mg protein; $P < 0.001$) compared with Ad.Egr-TNF.11D alone (2,688.24 ± 533.57 pg/mg protein). *Columns,* mean; *bars,* SEM.

induction by taxol were not feasible due to severe systemic toxicity at the doses employed in these studies. The results show that, like ionizing radiation, chemotherapeutic agents induce the production of TNF-α protein by tumors transduced with the Ad.Egr-TNF.11D vector.

Chemoinduction of Ad.Egr-TNF.11D Overcomes Doxorubicin and TNF-α Resistance in PC-3 and PRObXenografts

PC-3 tumors have been shown to be resistant to doxorubicin *in vivo* (48) and PC-3 cells resistant to TNF-α *in vitro* (data not shown). Based on previous studies demonstrating that radioinduction of Ad.Egr-TNF.11D produces significant antitumor effects in radioresistant tumors due to the destruction of the tumor microvasculature (21, 25, 26), we asked whether the combination of Ad.Egr-TNF.11D and doxorubicin would be effective in overcoming resistance to chemotherapy and/or TNF-α.

PC-3 tumors (initial mean tumor volume, 368 ± 22 mm^3, $n = 59$) were injected with Ad.Egr-TNF.11D and mice were treated with doxorubicin. The data obtained from two independent experiments were combined and are shown in Fig. 4. Mice in the control group (injected i.t. with viral buffer and i.p. with saline) and those in doxorubicin group (injected i.t. with viral buffer and i.p. with doxorubicin) exhibited equivalent tumor growth with mean volume increasing by 3-fold at day 23. Treatment with Ad.Egr-TNF.11D alone significantly reduced mean tumor volume beginning on day 9 ($P = 0.008$) and continuing to day 23 ($P = 0.005$) compared with the buffer-injected control group. The combination of Ad.Egr-TNF.11D and doxorubicin produced the greatest reduction in mean tumor volume, reaching a nadir (90% reduction) at day 13 that persisted for the duration of the experiment. A significant difference between the Ad.Egr-TNF.11D alone group and the combination group was detectable on day 16 ($P = 0.025$) and continued until day 23 ($P = 0.006$; Fig. 4A). These results indicate that the combination of Ad.Egr-TNF.11D and doxorubicin overcomes the lack of response to doxorubicin. Systemic toxicity was observed in 30% of mice exposed to doxorubicin. Importantly, these adverse effects were not increased with the combination of Ad.Egr-TNF.11D and doxorubicin.

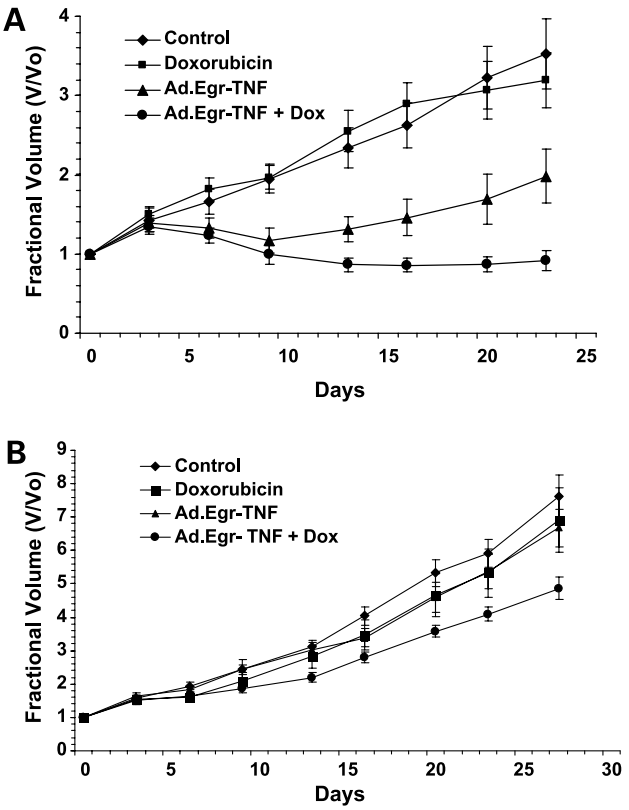

Figure 4. Xenograft regrowth studies. **A,** in PC-3 xenografts, combined treatment with Ad.Egr-TNF.11D and doxorubicin produced significant tumor regression compared with Ad.Egr-TNF.11D alone on *days 16* ($P = 0.025$), *20* ($P = 0.039$), and *23* ($P = 0.006$). **B,** in PRObxenografts, significant tumor regression was observed in the tumors receiving combined treatment with Ad.Egr-TNF.11D and doxorubicin compared with Ad.Egr-TNF.11D alone on *days 23* ($P = 0.027$) and *27* ($P = 0.015$). *Day 0,* first day of treatment. *Points,* mean; *bars,* SEM.

The effects of Ad.Egr-TNF.11D alone, doxorubicin alone, and the combination of both agents were studied in similar experiments done in PRObxenografts. We employed tumors with a mean volume of 318.3 ± 18 mm^3 ($n = 40$) at day 0. There was no difference in tumor growth delay at day 27 among the buffer-injected control group (mean fractional volume, 7.6), the doxorubicin alone group (mean fractional volume, 6.9), and the Ad.Egr-TNF.11D alone group (mean fractional volume, 6.7). Notably, treatment with Ad.Egr-TNF.11D and doxorubicin produced a significant reduction in mean fractional tumor volume compared with Ad.Egr-TNF.11D alone at day 23 (4.1 versus 5.4; $P = 0.027$). At day 27, tumors in the Ad.Egr-TNF.11D and doxorubicin group exhibited a 4.9-fold increase in fractional tumor volume compared with a 6.7-fold increase in the Ad.Egr-TNF.11D alone group ($P = 0.015$; Fig. 4B). These results suggest that combination treatment with Ad.Egr-TNF.11D and doxorubicin overcomes resistance to both doxorubicin and TNF-α. Toxicity, including weight loss and deaths, was observed in groups receiving doxorubicin alone; however, these effects were not increased with the addition of Ad.Egr-TNF.11D.

Combined Treatment of PC-3 Xenografts with Ad.Egr-TNF.11D and Doxorubicin Decreases Tumor Microvessel Density Compared with Either Treatment Alone

To study the effects of doxorubicin-mediated induction of Ad.Egr-TNF.11D on tumor angiogenesis, CD31-positive tumor vessels were counted on tissue sections from PC-3 tumors. Combined treatment with Ad.Egr-TNF.11D and doxorubicin reduced the number of vessels per high-power field (5.35 ± 0.78) compared with the control group (7.89 ± 0.54; $P = 0.005$), the doxorubicin alone group (6.24 ± 0.35; $P = 0.069$), and the Ad.Egr-TNF.11D alone group (6.5 ± 0.43; $P = 0.057$). In the Ad.Egr-TNF.11D and doxorubicin treatment group (Fig. 5D), there were fewer vessels of all diameters and less branching when compared with tumors

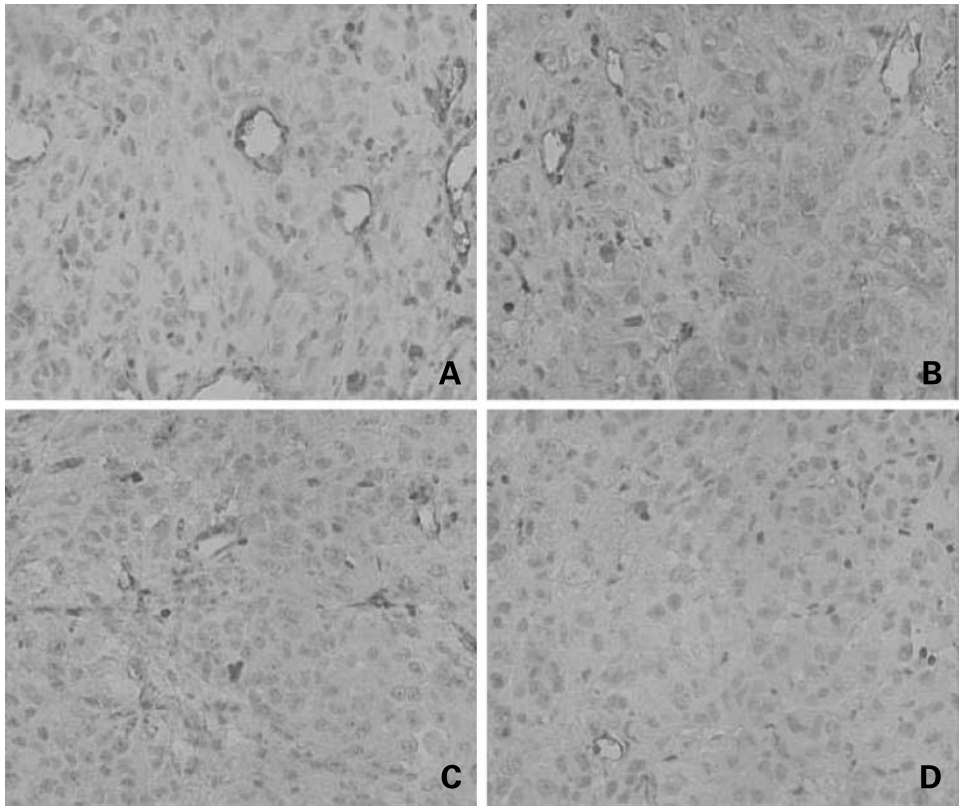

Figure 5. Visualization of tumor vasculature using anti-CD31 immunohistochemistry. Micrographs are representative PC-3 xenografts (day 27) following treatment with Ad.Egr-TNF.11D and doxorubicin. Microvessels were visualized in paraffin-embedded tissue sections using the avidin-biotin peroxidase technique. **A,** untreated control; **B,** doxorubicin; **C,** Ad.Egr-TNF.11D alone; **D,** Ad.Egr-TNF.11D + doxorubicin.

from the control group (Fig. 5A), the doxorubicin alone treatment group (Fig. 5B), and the Ad.Egr-TNF.11D alone treatment group (Fig. 5C). These results indicate that activation of Ad.Egr-TNF.11D enhances treatment with doxorubicin, at least in part, by inhibiting angiogenesis.

Discussion

We report induction of the Ad.Egr-TNF vector by different classes of widely used chemotherapeutic agents. We also show that tumor resistance to doxorubicin can be altered by combining doxorubicin with the Ad.Egr-TNF.11D vector. Moreover, we show that tumor microvessel density is decreased with the combination of doxorubicin and Ad.Egr-TNF.11D as compared with doxorubicin alone or Ad.Egr-TNF.11D alone. These data suggest that the alteration of doxorubicin resistance by the combination of doxorubicin and Ad.Egr-TNF.11D is due in part to the inhibition of tumor angiogenesis. TNF-α induces the activity and release of angiostatin-converting enzymes (49). In this regard, angiostatin is elevated in the plasma of tumor-bearing mice treated with Ad.Egr-TNF.11D (49). Additionally, we (49) and others (50) have reported that human tumor cells, including PC-3 cells, produce enzymes capable of converting plasminogen to angiostatin. Angiostatin is reported to be an effective antitumor agent when combined with DNA-damaging agents through the inhibition of tumor angiogenesis (51). Taken together, these data suggest that the antitumor activity of doxorubicin and Ad.Egr-TNF.11D may be mediated by the inhibitory effects of angiostatin and doxorubicin on tumor angiogenesis. Although our results suggest an antiangiogenic effect of combined treatment with doxorubicin and Ad.Egr-TNF.11D, we cannot exclude the possibility that TNF-α directly sensitizes tumor cells to doxorubicin killing or vice versa. PC-3 cells are p53 null (44) and express chemoresistance genes (45, 46). Potential mechanisms of chemoresistance of PROb cells have not been reported. In the current studies, PC-3 cells are more sensitive to the cytotoxic effects of combined treatment with Ad.Egr-TNF.11D plus doxorubicin compared with PROb cells (30% versus 85% survival at 300 μmol/L). These findings may explain the greater antitumor effects of Ad.Egr-TNF.11D plus doxorubicin in PC-3 tumors compared with PROb tumors. Our findings are similar to those of Park et al. (47), who reported enhanced antitumor effects following combined treatment with cisplatin and Ad.Egr-TNF.11D.

Egr-1 gene transcription has been shown previously to be induced by ROI-producing agents such as ionizing radiation and H_2O_2 through the activation of CArG sequences (43–42, 52, 53). The activation of Ad.Egr-TNF.11D by all of the chemotherapeutic compounds studied in the present work was altered by *N*-acetylcysteine. Taken together, these data show that activation of the Egr-TNF construct is mediated, at least in large part, by ROIs produced by these chemotherapeutic agents. The induction of Egr-1 by agents

that produce ROIs is consistent with reports that changes in cellular oxidation/reduction regulate the activation of several transcription factors including c-Fos and c-Jun (43, 54–56). Mitomycin C, vincristine, topotecan, resveratrol, and cisplatin have also been shown to activate *egr-1* transcription (57, 47). The available data on *egr-1* gene induction, considered together with the results reported herein, suggest that chemoinducible gene therapy based on control of transgene expression by free radical production may be applicable to diverse chemotherapeutic agents. It is noteworthy that several studies, including our own, report constitutive activity of the Egr-1 promoter. Although low levels of TNF-α are produced by the Ad.Egr-TNF vector, toxicity has not been observed in animal or human studies.

Chemoinducible Ad.Egr-TNF.11D gene therapy is potentially applicable to patients with tumors that are not amenable to cure with surgery and/or radiotherapy or patients with tumors that have failed conventional treatments in which the tumor mortality is related to local or regional extension. Examples include head and neck cancers, cervical cancer, gliomas, and some subsets of patients with lung, colorectal, hepatobilliary, pancreatic, and ovarian cancers. In addition, chemoinducible Ad.Egr-TNF.11D gene therapy may be a less toxic alternative to the treatment of locally symptomatic sarcomas and melanomas in which limb perfusion is currently a treatment option. Successful treatment of widely metastatic disease by chemoinducible gene therapy requires targeted delivery of the toxic gene product and control of therapeutic gene expression to selectively enhance tumor kill. The development of tumor targeted delivery systems such as genetically engineered viruses or nanoparticles may expand the applications of chemoinducible gene therapy to cancers that have metastasized beyond the primary tumor site. Finally, the inducibility of Ad.Egr-TNF.11D by broad classes of anticancer agents supports extensive applicability of this approach in clinical oncology.

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