**E1A gene therapy inhibits angiogenesis in a Ewing’s sarcoma animal model**

Zhichao Zhou,¹ Rong-Rong Zhou,¹ Hui Guan,¹ Corazon D. Bucana,² and Eugenie S. Kleinerman¹,²

¹ Division of Pediatrics and ² Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX

Abstract

We assessed vascular endothelial growth factor (VEGF) expression in four different human Ewing’s sarcoma cell lines (TC71, SK-ES, RD, and A4573) and in tumors in nude mice induced following s.c. injection of TC71 cells. Three of the four cell lines (TC71, SK-ES, and A4573) expressed significantly higher levels of VEGF than did normal human osteoblasts. Transfection of the adenovirus type 5 early region 1A (E1A) gene into TC71 cells down-regulated VEGF expression in vitro. In the mice bearing TC71 cell tumors, intratumoral injections of an adenoviral vector containing the E1A gene (Ad-E1A) decreased VEGF expression, inhibited tumor growth, and increased the survival rates in comparison with the mice given injections of PBS or an adenoviral vector containing β-galactosidase (Ad-β-gal). E1A gene therapy also significantly reduced blood vessel density and induced cell apoptosis in the tumors. These results demonstrate that E1A gene therapy inhibits angiogenesis, most likely by suppression of VEGF expression. Thus, E1A gene therapy may be a new therapeutic approach for Ewing’s sarcoma. (Mol Cancer Ther. 2003;2(12):1313–1319)

Introduction

Ewing’s sarcoma is a relatively rare tumor with limited therapeutic options. The majority of patients are initially responsive to combination chemotherapy, surgery, and/or radiation. However, 40–60% of patients relapse during the first 2 years. Relapsed Ewing’s sarcoma is usually resistant to salvage chemotherapy and is often highly aggressive. Despite multiple changes in the adjuvant chemotherapeutic regimens used to treat Ewing’s sarcoma, the 2-year metastasis-free survival rate has remained at 40% and the 3-year overall survival rate at 50% for the past 10 years (1, 2).

Second remissions are rare as Ewing’s sarcoma tumors usually do not respond to chemotherapy. Better understanding of the tumor biology of Ewing’s sarcoma and the mechanism involved in tumor growth, such as angiogenesis, may uncover new therapeutic approaches.

Angiogenesis is a crucial step in the continued growth of solid tumors. Rapidly growing primary and metastatic tumors are generally very vascular with blood vessel expanding as the tumors increases in size (3). Several factors are involved in the angiogenesis pathway. Vascular endothelial growth factor (VEGF) is one of the important regulators in tumor angiogenesis and is overexpressed in various solid tumors. Here we demonstrated that three of four human Ewing’s sarcoma cell lines overexpressed VEGF. Thus, we postulated that VEGF may be a therapeutic target for the treatment of Ewing’s sarcoma and investigated the use of a tumor suppressor gene to modulate VEGF expression.

The adenovirus type 5 early region 1A (E1A) gene is an important tumor suppressor gene. E1A gene therapy inhibited tumor growth, induced apoptosis, and enhanced the sensitivity of tumors to chemotherapy (4, 5). Phase I and phase II clinical trials with E1A gene therapy were performed in patients with metastatic breast, ovarian, and head and neck cancer. E1A was shown to inhibit tumor growth by a mechanism involving the down-regulation of HER-2/neu in overexpressing tumors. Recent studies have suggested that the antitumor activity of the E1A gene is not limited to inhibition of HER-2/neu overexpression (6, 7). Transcriptional repression of many oncoproteins may be involved in conversion of tumor cells to an epithelial phenotype and the induction of apoptosis in both p53-dependent and p53-independent mechanisms. E1A gene therapy also increased the sensitivity of tumor cells to chemotherapy (8), and mediates an antiangiogenic effect in ovarian tumors (9). Recent studies have indicated a link between the overexpression of HER-2/neu and an increased expression of VEGF in human tumor cells (10, 11). Hereceptin, a HER-2/neu monoclonal antibody, was demonstrated to have an antiangiogenic effect (12).

We have previously demonstrated that TC71 human Ewing’s sarcoma cells express high levels of HER-2/neu. Ad-E1A (adenoviral vector containing the E1A gene) gene therapy down-regulated HER-2/neu overexpression, inhibited tumor growth, and enhanced the sensitivity of the tumors to chemotherapy both in vitro and in vivo (13, 14).

In the present study, we investigated the VEGF expression in four different Ewing’s sarcoma cell lines, and determined the effect of E1A gene therapy on tumor VEGF expression and angiogenesis using an Ewing’s sarcoma mouse model. Our data demonstrated that E1A gene therapy down-regulated VEGF expression in vitro and in vivo, inhibited tumor angiogenesis and tumor growth, and prolonged animal survival.
Materials and Methods

Cell Lines
Normal human osteoblast cells were purchased from Clonetics, Corp. (San Diego, CA) and maintained in the special medium provided by Clonetics. TC71 human Ewing’s sarcoma cells, which were kindly provided by Dr. T. Triche (University of Southern California, Los Angeles, CA), were cultured in Eagle’s modified essential medium with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 1 mM sodium pyruvate, 1× nonessential amino acid, and 2× MEM vitamin solution (Life Technologies, Inc. Grand island, NY). The SK-ES and RD human Ewing’s sarcoma cells were obtained from American Type Culture Collection (Manassas, VA). SK-ES cells were cultured in McCoy’s 5A medium with 15% FBS and the RD cells were cultured in RPMI 1640 with 15% FBS. A4573 human Ewing’s sarcoma cells, which were a generous gift from Dr. V. Soldatenkov (Georgetown University Medical Center, Washington, D.C.), were cultured in Eagle’s modified essential medium. All of the cells were screened by a Mycoplasma Plus PCR Primer Set (Stratagene, La Jolla, CA) and found to be free of Mycoplasma.

Recombinant Adenovirus
Ad-E1A is an adenovirus type 5 which contains E1A but lacks E1B and E3 (15). The control adenovirus, Ad-β-gal, is an adenovirus type 5-based vector that lacks E1A, E1B, and E3 but contains β-galactosidase. Both of these recombinant replication-deficient adenoviral vectors were propagated in human embryonic kidney 293 cells as previously described (16). The viruses were purified twice using cesium chloride-gradient ultracentrifugation and then dialyzed and titrated using the standard methods. The cells were infected with adenovirus at 1 plaque-forming units (pfu) per cell for 48 h and then treated as indicated for various experiments.

Northern Blot
Total RNA was isolated from the cells using Trizol reagents (Life Technologies). Twenty micrograms of RNA were subjected to electrophoresis on 1% formaldehyde-reagents (Life Technologies). Twenty micrograms of RNA for various experiments.

cells were infected with adenovirus at 10 plaque-forming

dialyzed and titrated using the standard methods. The
cesium chloride-gradient ultracentrifugation and then
cultured in human embryonic kidney 293 cells as previously
binant replication-deficient adenoviral vectors were prop-
ated nick end labeling (TUNEL) assay.

Immunohistochemical Analysis
Tumor tissue sections were taken from mice bearing
TC71 Ewing’s sarcoma. The sections were analyzed by
routine pathology using H&E staining. Frozen sections
were fixed with acetone and then incubated in 3% H 2O2 in
expression of VEGF for each cell line was calculated and
adjusted by 18S internal control.

In Vivo Animal Studies
Four- to 5-week-old specific pathogen-free athymic (T-cell-deficient) nude mice were purchased from Charles River Breeding Laboratories (Kingston, MA). The mice were housed in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care and in accordance with the current regulations and standards of the United States Department of Agriculture and Department of Health and Human Services and the National Institutes of Health. The mice were housed for 1–2 weeks before any of the experiments began.

TC71 Ewing’s sarcoma cells in mid-log-growth phase were harvested by trypsinization. The nude mice were given subcutaneous injections of 2 × 106 cells in 0.1 ml HBSS (4°C). Tumors could be detected 1 week after injection. The tumor-bearing mice were then given intratumoral injections of Ad-E1A (1010 pfu/ml), Ad-β-gal (1010 pfu/ml), or PBS as a control. Total volume in 50 μl was injected in three different tumor sites. Injection was performed twice per week for 2 weeks. Ten mice were used for each group. Each experiment was repeated three times. The tumors were measured every 3–5 days with calipers, and their diameters were recorded. Tumor volumes were calculated using the formula 1/2 × ab2, where a is the longer diameter and b is the shorter diameter and expressed in cubic millimeters. The duration of survival was recorded. Each mouse was sacrificed when its tumor exceeded 2 cm × 2 cm. Tumor tissue was collected from each group and analyzed for VEGF, CD31 expression by immunohistochemical staining and for apoptosis by terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay.

Immunohistochemical Analysis
Tumor tissue sections were taken from mice bearing
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routine pathology using H&E staining. Frozen sections
were fixed with acetone and then incubated in 3% H2O2 in
methanol for 10 min to block endogenous peroxidase. We
then incubated them in 5% normal horse serum plus 1% normal goat serum in PBS for 20 min to block nonspecific protein. Expression levels of the CD31 gene were detected
in blood vessels using rat anti-mouse CD31 as the primary antibody (PharMingen, San Diego, CA), and goat anti-rat horseradish peroxidase as the secondary antibody, then incubated with chromogen diaminobenzidine. We counted the number of blood vessels in five random microscopic fields and then determined the mean. The analysis was performed in triplicate, and the results were expressed as the mean of three independent experiments. The VEGF expression was detected by incubating the tissue sections with rabbit anti-human VEGF antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) as the primary antibody and goat anti-rabbit IgG with horseradish peroxidase (Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) as the secondary antibody. Gill’s hematoxylin was used as a counterstain.

**TUNEL Assay**

TUNEL assay was performed to detect apoptotic cells. Frozen sections were fixed with 4% methanol-free formaldehyde solution in PBS for 10 min and then washed three times with PBS. The tissues were permeabilized in 20 μg/ml proteinase K solution for 10 min at room temperature and equilibrated in equilibration buffer for 10 min after which the slides were rinsed three times with PBS. The DNA fragments were labeled with fluorescein-12-dUTP in terminal deoxynucleotidyl transferase incubation buffer (Promega) in a humidified chamber (37°C for 60 min) to avoid exposure to light. The reactions were terminated by transferring the slides to 2 × SSC buffer (0.3 M NaCl, 0.03 M sodium citrate, pH 7.0) for 15 min and washing them in PBS to remove unincorporated fluorescein-12-dUTP. The slides were then counterstained with 4',6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA) to provide a blue background. The green fluorescence of apoptotic cells (fluorescein-12-dUTP) can be detected with a fluorescence microscope at 520 nm.

**Statistical Analysis**

We used the two-tailed Student t test to statistically evaluate the tumor volumes and numbers of blood vessels. A P value of <0.05 was considered statistically significant difference. We used the Log-rank test to analyze the survival curve. A P value of <0.05 was considered statistically significant.

**Results**

**VEGF Expression in Ewing’s Sarcoma Cell Lines**

Comparison of the levels of VEGF expression in the four human Ewing’s sarcoma cell lines, TC71, SK-ES, RD, and A4573, to the VEGF expression levels in normal human osteoblast cells by Northern blot and RT-PCR, is shown in Fig. 1. The TC71, SK-ES, and A4573 cell lines expressed higher levels of VEGF than normal osteoblasts did. In the TC71 cells, VEGF expression was 4.1 times greater (by Northern blot) and 3.1 times greater (by RT-PCR) than it was in normal osteoblasts. The VEGF expression levels were 3.3 and 3.2 times greater in SK-ES and A4573 cells, respectively, than in normal osteoblasts as determined by Northern blot. The VEGF expression was only slightly higher in RD cells than in normal osteoblasts. VEGF expression in vivo of Ewing’s sarcoma tumors was also determined. Immunohistochemistry staining (Fig. 2A1) showed higher levels of VEGF in the TC71 tumors. These results demonstrated that three of four Ewing’s tumor cell lines overexpressed VEGF. Furthermore, tumor tissue induced in vivo by TC71 cells also expressed higher levels of VEGF.

**E1A Down-Regulated VEGF Expression**

Transfection of the E1A gene into TC71 cells using an adenoviral vector (Ad-E1A) significantly reduced the VEGF expression as measured by both Northern blot and RT-PCR (Fig. 3). VEGF expression was decreased by 40% following transfection of the E1A gene compared with the TC71 control cells. In contrast, transfection of TC71 cells with Ad-β-gal did not significantly alter VEGF expression. The intratumoral injection of Ad-E1A was also able to down-regulate VEGF expression in vivo. Immunohistochemistry staining of tumor samples demonstrated that VEGF expression was significantly reduced in the tumor treated with Ad-E1A (Fig. 2A2) compared with untreated.
control tumor (Fig. 2A1) or tumor treated with Ad-β-gal (Fig. 2A3). Since other investigators have demonstrated a link between HER-2/neu expression and hypoxia inducible factor-1α (HIF-1α) (18), we also investigated whether the down-regulation of HER-2/neu and VEGF induced following E1A transfection affected HIF-1α expression. Following treatment of TC71 cells with Ad-E1A, both VEGF and HER-2/neu expression decreased. By contrast, HIF-1α protein expression was not significantly altered (data not shown).

**Effect of Ad-E1A Gene Therapy on Vessel Density**

We next determined the effect of E1A gene therapy on tumor vessel density in vivo. Immunohistochemical staining using the CD31 antibody indicated that the intratumoral injections of Ad-E1A resulted in a significant reduction in tumor vessels (Fig. 2B2). In contrast, the Ad-β-gal treatment had little or no effect on vascular density.

**Figure 2.** E1A gene therapy down-regulates VEGF expression, reduces tumor blood vessel density, and induces tumor cell apoptosis in vivo. A, immunohistochemical staining with a VEGF antibody was performed in an untreated TC71 tumor (A1) and in tumors treated with Ad-E1A (A2) or Ad-β-gal (A3). B, CD31 immunohistochemical staining was performed to detect blood vessels in tumor samples from control mice (B1) and from mice treated with Ad-E1A (B2) or Ad-β-gal (B3). C, apoptotic cells were detected in the tumor tissue by TUNEL assay in untreated specimens (C1) and in specimens treated with Ad-E1A (C2) or Ad-β-gal (C3).

**Figure 3.** E1A gene transfer down-regulates VEGF expression in TC71 cells. The total RNA was extracted from TC71 cells following treatment with PBS (TC71), Ad-E1A (+Ad-E1A), or Ad-β-gal (+Ad-β-gal). Northern blot (left panel) and RT-PCR (right panel) were performed. Each band was analyzed by densitometry and the value was adjusted by GAPDH or 18S loading control. The relative fold expression of VEGF for each band was calculated and compared with that of the TC71 control cells. VEGF expression in the TC71 cells was reduced by 40% following transfection of the E1A gene.
(Fig. 2B3). The mean number of blood vessels (±SE) in the control tumors was 75 ± 8 compared with 39 ± 3 in Ad-E1A-treated tumors (Fig. 4, P < 0.05). Ad-β-gal treatment did not significantly alter the mean number (71 ± 7) of blood vessels (Fig. 4). These results indicate that E1A gene therapy not only down-regulated VEGF expression but also reduced the tumor vascular density. These data suggest that E1A gene transfer has an antiangiogenic effect in Ewing’s sarcoma in addition to its effect on HER-2/neu.

**Ad-E1A Induced Apoptosis in Vivo**

To explore the mechanism by which E1A suppressed tumor growth in vivo, we examined the apoptosis in tumor tissues after E1A gene therapy. Tumors treated with Ad-E1A (Fig. 2C2) contained more apoptotic cells (P < 0.05) compared with the control tumor (Fig. 2C1) or tumor treated with Ad-β-gal (Fig. 2C3). The average number of TUNEL-positive cells/high power field (±SE) in the Ad-E1A-treated tumors was 142 ± 22 compared with 70 ± 10 in the Ad-β-gal-treated tumor and 49 ± 16 in the control tumors. These results indicated that E1A-induced apoptosis may play a role in the suppression of Ewing’s sarcoma tumor growth seen following E1A gene therapy.

**Ad-E1A Gene Therapy Inhibited Tumor Growth and Increased Survival Rates**

We have demonstrated that E1A gene transfer down-regulated VEGF expression in vitro and in vivo. We next determined whether E1A-induced down-regulation of VEGF expression affects tumor growth and survival rates in the mice. As shown in Fig. 5, the mean tumor volume was significantly lower in the mice treated with Ad-E1A than the mice in the untreated control groups or Ad-β-gal groups (P < 0.05). Thirty-five days after treatment, the mean tumor volume in the group treated with Ad-E1A was significantly smaller than those in the control groups.

Survival rates (Fig. 6) in the E1A-treated mice were also significantly longer than those of the PBS- or Ad-β-gal-treated mice (P < 0.05). These results suggest that E1A gene therapy inhibits Ewing’s sarcoma tumor growth in vivo and increased survival rates.

**Discussion**

Overexpression of angiogenic genes such as VEGF has been shown to correlate with enhanced tumorigenicity and metastatic potential. VEGF is important to the growth of many solid tumors conferring survival advantage by inducing vascular formation. Serum levels of VEGF were found to be increased in patients with brain, melanoma, breast, renal, and gastrointestinal malignancies (19–28).
High levels of VEGF correlate with both tumor vascularity and poor prognosis (21, 22). Investigators have also reported that the preoperative levels of VEGF in the sera of patients with colorectal cancer are predictive of stage and clinical outcome (29). High levels of VEGF have also been found in the sera of patients with Ewing’s sarcoma (30).

Our data demonstrate that VEGF is overexpressed in three of four tested Ewing’s sarcoma cell lines. E1A gene transfer using an adenoviral vector down-regulated VEGF expression in vitro and in vivo. Intratumoral injections of E1A resulted in tumor cell apoptosis with a decrease in tumor vascularity and growth. In addition, treatment with intratumoral E1A resulted in increased animal survival. By contrast, intratumoral injections of Ad-β-gal had no effect on tumor apoptosis (Fig. 2C), tumor vascularity (Fig. 2B3), tumor size, or long-term survival (Figs. 5 and 6).

We have previously demonstrated that Ewing’s sarcoma cells overexpress HER-2/neu. E1A gene transfer down-regulated HER-2/neu expression and up-regulated topoisomerase IIα expression, resulting in the increasing cell sensitivity to etoposide and Adriamycin (13, 14). Our present study indicates that in addition to its effect on HER-2/neu and topoisomerase IIα, E1A gene therapy also has antiangiogenic activity most likely achieved through inhibition of VEGF expression. Other studies have also shown a link between the overexpression of HER-2/neu and high levels of VEGF (18). Therefore, E1A gene therapy may mediate tumor regression by shutting down the expression of both these genes. Whether this is a direct effect of E1A on the VEGF promoter as was shown with topoisomerase IIα expression (13) or indirect effect is unclear. While Laughner et al. (18) demonstrated a correlation between HER-2/neu- and HIF-1α-mediated VEGF expression, we were unable to detect an alteration in HIF-1α expression following E1A transfection in TC71 cells.

Our data indicated that E1A gene therapy may provide a new approach for the treatment of Ewing’s sarcoma. Clinical trials with E1A gene therapy have demonstrated safety in patients with metastatic breast, ovarian, and head and neck cancer (6, 31). Yoo et al. (6) reported that no severe drug-related adverse events were observed and no mild or moderate dose-dependent adverse events were noted in the phase I clinical trial of intratumoral liposome E1A gene therapy in patients with recurrent breast and head and neck cancer. Because of the limited therapeutic options available for relapsed patients, Ewing’s sarcoma may be an additional tumor type to consider for phase II E1A gene therapy trials.

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References
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