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E1A Sensitizes *HER2/neu*-overexpressing Ewing's Sarcoma Cells to Topoisomerase II-targeting Anticancer Drugs[1]

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ABSTRACT

Overexpression of the *HER2/neu* oncogene is associated with tumorigenicity and drug resistance in many types of cancer. Three different human Ewing's sarcoma cell lines (TC71, RD, and A4573) were found to express high levels of the HER2/neu protein. Transduction of TC71 cells with the *E1A* gene using an adenoviral vector (Ad-*E1A*) down-regulated HER2/neu overexpression in those cells and increased cytostasis. *E1A*-induced apoptosis was demonstrated by both flow cytometric analysis and Western blot analysis using a poly(ADP-ribose) polymerase antibody. After transduction of the *E1A* gene into these cells, the sensitivity of these cells to VP-16 (etoposide) was enhanced 18-fold and to Adriamycin 5-fold. However, no change was seen in cisplatin sensitivity. *E1A* also significantly increased topoisomerase IIα protein expression, indicating that the up-regulation of topoisomerase IIα may be one of the mechanisms by which *E1A* enhanced the sensitivity to topoisomerase II-targeting anticancer drugs, such as VP-16 and Adriamycin, but not cisplatin. In summary, these studies demonstrated that Ad-*E1A* can down-regulate HER2/neu overexpression and up-regulate topoisomerase IIα expression in human Ewing's sarcoma cells, increasing their apoptosis rate and enhancing their sensitivity to VP-16 and Adriamycin.

INTRODUCTION

Ewing's sarcoma is the second most common malignant bone tumor in children (1). The majority of patients have microscopic metastases at diagnosis; the lung is the most common metastatic site (2). These patients initially respond to systemic chemotherapy plus surgery and/or radiation. However, at 2 years, 40–60% will, depending on the prognostic indicators, have a recurrence. Adverse prognostic indicators include large tumor volume or tumor length, tumor location, elevated serum lactic dehydrogenase, and metastatic spread to bone, lung, or bone marrow. A number of different chemotherapy regimens, including those comprising total-body irradiation and autologous bone marrow transplant, have been unsuccessful in increasing the survival rates at 2 and 3 years (3–7). Furthermore, relapsed patients are particularly resistant to second-line chemotherapy, leaving few treatment options.

The *HER2/neu* oncogene encodes a M_r 185,000 human epidermal growth factor receptor-2 transmembrane protein. Overexpression of HER2/neu has been found in ~30% of human breast and ovarian cancers (8, 9). Enhanced expression of HER2/neu increased the tumorigenicity and metastatic potential of human cancer cells (10, 11). Clinical studies indicate that overexpression of HER2/neu correlates with poor prognosis, shorter patient survival in osteosarcoma (12, 13). Because of the close association reported between overexpression of HER2/neu and resistance to chemotherapeutic agents (14, 15) and because of the poor disease-free survival rates associated with multiagent chemotherapy in Ewing's sarcoma, we investigated HER2/neu expression in several different Ewing's sarcoma cell lines. HER2/neu expression was high in all three of the Ewing's sarcoma cell lines examined.

Down-regulation of HER2/neu may be an important approach for cancer therapy (16, 17). The *E1A*[3] gene has been shown to be a tumor suppressor for cells that overexpress HER2/neu (18, 19). Transfer of the *E1A* gene results in transcriptional repression of *HER2/neu* via an effect on the *HER2/neu* promoter. *In vivo*, E1A gene transfer suppressed the dissemination of ovarian cancer cells that overexpressed HER2/neu and enhanced the *in vivo* sensitivity of the cells to paclitaxel (20, 21). In this study, we found that *E1A* down-regulated HER2/neu expression, inhibited tumor cell growth, and increased cellular sensitivity to topoisomerase II-directed anticancer drugs. These results suggest that *E1A* gene therapy aimed at down-regulation of HER2/neu may provide a new approach for the therapy of Ewing's sarcoma.

MATERIALS AND METHODS

Cell Lines. TC71 human Ewing's sarcoma cells, kindly provided by Dr. P. Pepe (University of Southern California, Los Angeles, CA), were cultured in Eagle's modified essential medium with 10% fetal bovine serum. SKRB-3 human breast cancer cells and RD human Ewing's sarcoma cells were obtained from American Type Culture Collection (Manassas, VA). A4573 human Ewing's sarcoma cells were a generous gift from Dr. Soldatenkov (Georgetown University Medical Center, Washington, DC) and were cultured in RPMI 1640 with 15% fetal bovine serum. Normal human osteoblast cells were purchased from Clonetics, Inc. (San Diego, CA) and were maintained in the special medium provided by Clonetics. All cells were free of *Mycoplasma* as screened by Gen-Probe (Gen-Probe, San Diego, CA).

Recombinant Adenovirus. Ad-*E1A* is an adenovirus type 5 that contains *E1A* but lacks *E1B* and *E3* (18). The control adenovirus, Ad-*β-gal*, is an adenovirus type 5-based vector that lacks *E1A, E1B,* and *E3* but contains β-gal. Both of these recombinant replication-deficient adenoviral vectors were propagated in 293 cells as described previously (22). The viruses were purified twice using cesium chloride-gradient ultracentrifugation and then dialyzed and titrated by the standard method. Cells were infected with adenovirus at 10 plaque-forming units/cell for 48 h and then treated as indicated for various experiments.

Cytostasis Assay. Cells were seeded into 96-well cell culture plates (5000/well) and allowed to adhere overnight before being infected with Ad-*E1A* or Ad-*β-gal*. Various concentrations of VP-16, Adriamycin, or cisplatin were then added in triplicate. The antiproliferative activity was determined 48 h later by the MTT assay as described previously (23).

Western Blot. Cells were set in 100-mm dishes (2×10^6 per dish) 1 day before treatment and then were infected with Ad-*E1A* or Ad-*β-gal* for 48 h. Cells were washed with cold PBS buffer and lysed in RIPA buffer (1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) containing the fresh protease inhibitor aprotinin (2 μg/ml), leupeptin (2 μg/ml), pepstatin A (1 μg/ml), and phenylmethylsulfonyl fluoride (100 μg/ml; Sigma Chemical Co., St. Louis, MO). The cells were then scraped and passed through a 21-gauge needle. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). After 50 μg of the protein were solubilized in sample buffer, the solution was boiled for 5 min before loading onto a 7.5% SDS-polyacrylamide gel and then transferred to a nitro-

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[3] The abbreviations used are: E1A, adenovirus type 5 early region 1A; β-gal, β-galactosidase; VP-16, etoposide; topoIIα, topoisomerase IIα; PARP, poly(ADP-ribose) polymerase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

cellulose membrane. The specific protein bands were detected with a human Her2/c-neu (Ab-3) monoclonal antibody (Calbiochem, San Diego, CA), E1A (adenovirus type 5) antibody, PARP antibody (BD PharMingen, San Diego, CA), human topoIIα antibody (TopoGEN, Columbus, OH), and β-actin monoclonal antibody (Sigma) using the ECL Western blotting analysis system (Amersham) according to the manufacturers' instructions. Densitometric analysis was performed, and values were normalized with β-actin loading control.

Flow Cytometry Analysis. Apoptotic cells were detected by flow cytometry as described previously (24). TC71 cells were treated as indicated and then were washed with PBS buffer twice; 10^5 cells were incubated with propidium iodide solution (50 μg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) for 2 h. Cells were analyzed with a FACScan (Becton Dickinson Co., Mountain View, CA).

RESULTS

High levels of HER2/neu protein were detected in three different Ewing's sarcoma cell lines (TC7, RD, and A4573; Fig. 1) compared with the level in normal human osteoblast cells (NH Osteoblast). SKBR-3, a human breast cancer cell line well known to overexpress HER2/neu, was used as the positive control. Using HER2/neu expression in the normal human osteoblast cells as the baseline, the HER2/neu expression was 20.5-fold higher in TC71 cells, 11.2-fold higher in RD cells, and 13.9-fold higher in A4573 cells. We concluded that all three Ewing's sarcoma cell lines overexpress HER2/neu.

Effect of *E1A* on HER2/neu Expression. To determine whether *E1A* could down-regulate HER2/neu overexpression, TC71 cells were infected with Ad-*E1A* or control Ad-*β-gal*. HER2/neu and E1A expression were determined 48 h later (Fig. 2). TC71 cells infected with Ad-*E1A* expressed very high levels of E1A and had significantly reduced HER2/neu expression. The densitometric analysis suggested that HER2/neu expression was 80% lower in the *E1A*-infected TC71 cells than in the TC71 control cells. In contrast, TC71 cells infected with Ad-*β-gal* did not express E1A and had no significant change in the expression of HER2/neu.

Effect of HER2/neu on Cell Growth and Sensitivity to Chemotherapeutic Agents. Infection of TC71 cells with Ad-E1A inhibited cell growth by 25% (Fig. 3). By contrast, infection with Ad-*E1A* did not increase cytostasis in normal HER2/neu-expressing human osteoblast cells compared with those infected with Ad-*β-gal*. Down-regulation of HER2/neu overexpression by E1A resulted in inhibition of TC71 tumor cell growth. *E1A* also induced an 18-fold increase in the sensitivity of TC71 cells to VP-16 (Fig. 4). After transduction with the *E1A* gene, the IC_{50} was reduced from 1500 to 80 nM. Ad-*β-gal* did not significantly change the sensitivity of the cells to VP-16. The sensitivity of TC71 cells to Adriamycin was also increased, with a reduction in the IC_{50} from 90 to 18 nM (Table 1). By contrast, the sensitivity of TC71 cells to cisplatin did not significantly change after infection with Ad-*E1A*.

We next quantified the apoptosis of TC71 cells, both control and *E1A*-transduced, after exposure to VP-16 (Fig. 5). The percentage of sub-G_1 phase cells was only 2.4% in the control cells, 9.5% in cells treated with Ad-E1A, and 7.1% in cells treated with low-dose VP-16 (0.3 μM). The percentage of apoptotic cells in the cells treated with the same dose of VP-16 (0.3 μM) after infection with Ad-*E1A* was significantly increased to 29.3%. By contrast, Ad-*β-gal* did not significantly increase the rate of apoptosis induced by VP-16. These results were supported by Western blot analysis of PARP cleavage. PARP is a M_r 116,000 nuclear chromatin-associated enzyme. During cell apoptosis, PARP is cleaved from the M_r 116,000 intact form into the M_r 85,000 fragment. This cleaved band is a marker of apoptosis.

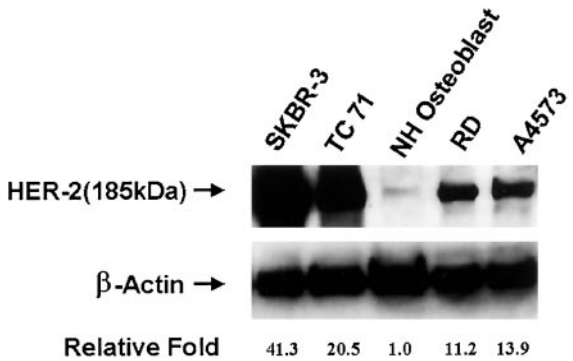

Fig. 1. HER2/neu expression in three different human Ewing's sarcoma cell lines. Proteins were extracted from three different human Ewing's sarcoma cell lines, TC71 (*Lane 2*), RD (*Lane 4*), and A4573 (*Lane 5*); from SKBR-3 human breast cancer cells (*Lane 1*); and from normal human osteoblast cells (*Lane 3*) and then subjected to electrophoresis on SDS-polyacrylamide gel. HER2/neu protein (M_r 185,000, *top panel*) and β-actin (M_r 42,000, *bottom panel*) were detected using the human HER2/neu (Ab-3) monoclonal antibody and β-actin antibody. Densitometric analysis was performed for each band, and values were normalized with a β-actin loading control. HER2/neu expression in normal human osteoblast cells was used as the baseline. The relative increase in the expression of HER2/neu for each cell line was calculated in comparison with that of normal human osteoblast cells.

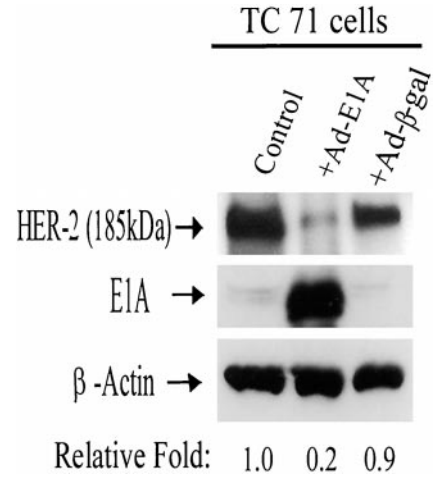

Fig. 2. Ad-*E1A* down-regulated HER2/neu expression. TC71 cells were treated with Ad-*E1A* (*Lane 2*), Ad-*β-gal* (*Lane 3*), or PBS as control (*Lane 1*) for 48 h. Proteins were extracted and subjected to electrophoresis on SDS-polyacrylamide gel. Specific antibodies were used for detection of HER2/neu (M_r 185,000), E1A (M_r 46,000), and β-actin (M_r 42,000) protein expression. Densitometric analysis was performed and adjusted by a β-actin loading control. The relative level of expression of HER2/neu is expressed in comparison to that in TC71 control cells.

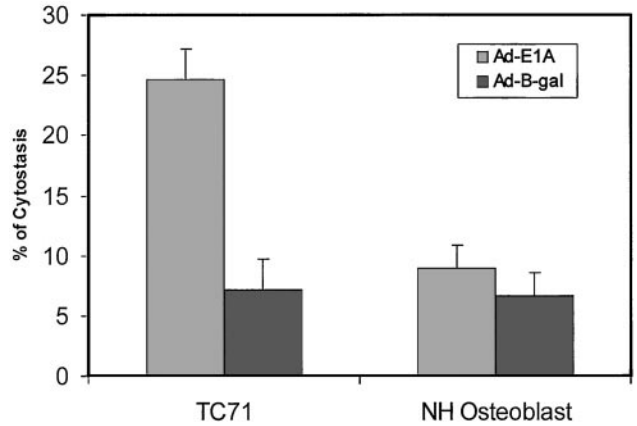

Fig. 3. Down-regulation of HER2/neu expression by *E1A* inhibited cell growth. TC71 cells and normal human osteoblast cells were incubated with Ad-*E1A*, Ad-*β-gal*, or PBS as control for 48 h. MTT assays were then performed, and cytostasis was quantified. The results were calculated as percentages of cytostasis compared with levels in control cells. The values were averaged from three different experiments. *Bars*, SD.

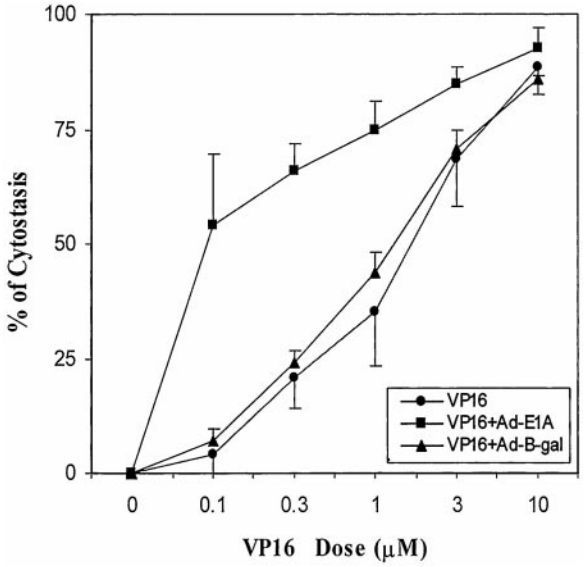

Fig. 4. Effects of Ad-*E1A* on cell sensitivity to VP-16. TC71 cells were incubated with Ad-*E1A*, Ad-*β-gal*, or PBS for 48 h and then treated with different doses of VP-16. Each result was calculated as a percentage of cytostasis compared with that detected in control cells using the MTT assay. The values are averaged from three different experiments. The IC_{50} is calculated as the dose that inhibited 50% of cell growth. *Bars*, SD.

Table 1 *Effect of Ad-E1A on sensitivity of TC71 cells to VP-16, Adriamycin, and cisplatin*[a]

Drugs	IC_{50} for drug only	IC_{50} for drug with Ad-E1A	Degree of sensitivity enhancement
VP-16	1500 nM	80 nM	18-fold
Adriamycin	90 nM	18 nM	5-fold
Cisplatin	630 nM	390 nM	1.6-fold

[a] TC71 cells were incubated with Ad-*E1A*, Ad-*β-gal*, or PBS for 48 h and then treated with different doses of VP-16, doxorubicin, or cisplatin. The results were calculated as percentages of cytostasis compared with control cells using the MTT assay. The values are the averages of results from three different experiments. The IC_{50} is calculated as the dose that inhibited 50% of cell growth. The degree of enhancement was calculated as the IC_{50} for drug only divided by the IC_{50} for drug in cells treated with Ad-*E1A*.

As shown in Fig. 6, the M_r 85,000 fragment was significantly more prominent in TC71 cells treated with Ad-*E1A* and VP-16 than in TC71 control cells, cells treated with VP-16 alone, or cells treated with Ad-*β-gal* and VP-16.

Effect of *E1A* Gene Transfer on topoIIα. topoIIα is the enzyme target for VP-16 and one of the targets for Adriamycin. By contrast, cisplatin is not considered to be a topoII-reactive drug. Because *E1A* gene transfer increased both VP-16 and Adriamycin sensitivity but had no significant effect on cisplatin sensitivity, we hypothesized that *E1A* gene transfer may affect not only HER2/neu but also topoIIα. As shown in Fig. 7, topoIIα protein levels were significantly increased in TC71 cells after exposure to Ad-*E1A* but not in cells exposed to Ad-*β-gal*.

DISCUSSION

Overexpression of the *HER2/neu* gene is correlated with enhanced tumorigenicity, enhanced metastatic potential, poor patient prognosis, and decreased chemosensitivity in many types of cancer, including breast, ovarian, lung, and stomach carcinomas (25, 26). This gene was also reported recently to be a potential prognostic indicator for osteosarcoma (13). The study presented here demonstrated that the *HER2/neu* oncogene is overexpressed in three different Ewing's sarcoma cell lines. *E1A* gene transfer using an adenoviral vector resulted in down-regulation of HER2/neu, decreased cell growth, and increased cellular sensitivity to VP-16 and Adriamycin but not cisplatin. Down-regulation of HER2/neu has been shown to increase apoptosis in tumor cells that overexpress the oncogene. We demonstrated here a similar increase in cell apoptosis and cytostasis after HER2/neu down-regulation.

Increased cell sensitivity to chemotherapy after *E1A* gene transfer has been demonstrated in several different tumor cell lines (27). However, the mechanism by which *E1A* enhances drug activity is not

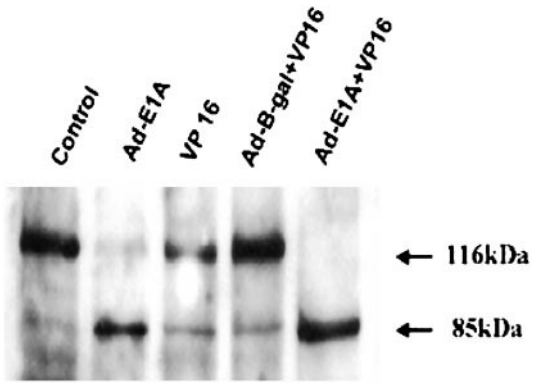

Fig. 6. Apoptosis of TC71 cells by PARP cleavage. TC71 cells were incubated with Ad-*E1A*, Ad-*β-gal*, or PBS for 48 h and then treated with VP-16. Proteins were extracted and subjected to electrophoresis on SDS-polyacrylamide gel. Anti-PARP monoclonal antibody was used to detect the intact (M_r 116,000) and cleaved (M_r 85,000) PARP forms.

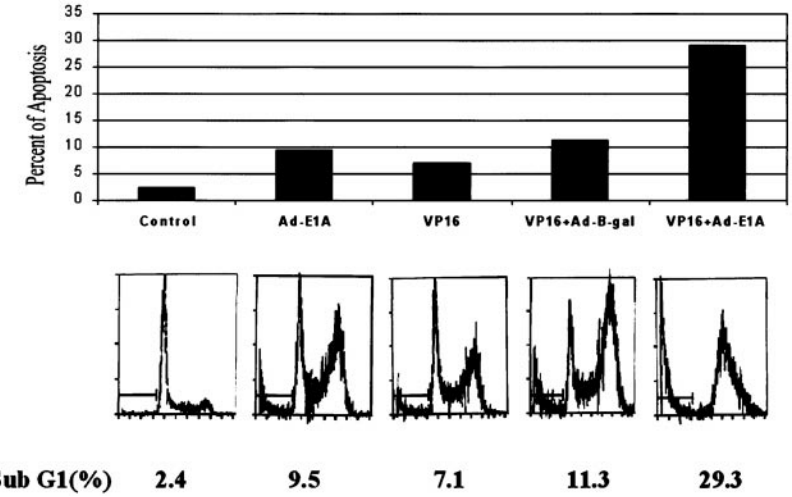

Fig. 5. Ad-*E1A* increased the apoptosis induced by VP-16. TC71 cells were incubated with Ad-*E1A*, Ad-*β-gal*, or PBS for 48 h and then treated with 0.3 μM VP-16. Cells were washed with cold PBS and incubated in the buffer; the percentage of cells undergoing apoptosis was quantified by using flow cytometry.

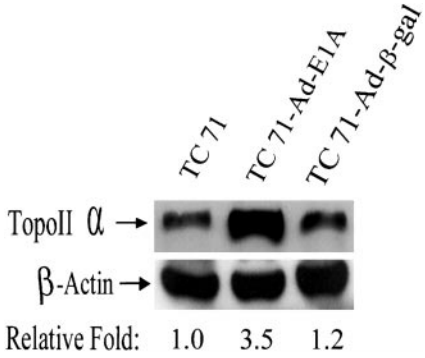

Fig. 7. Ad-*E1A* increased topoIIα protein expression. TC71 cells were incubated with Ad-*E1A* (*Lane 2*), Ad-*β-gal* (*Lane 3*), or PBS (*Lane 1*) for 48 h. Proteins were extracted and subjected to electrophoresis on SDS-polyacrylamide gel. Specific antibodies were used to detect topoIIα (M_r 170,000) and β-actin (M_r 42,000). Densitometric analysis was performed and adjusted by β-actin loading control. The relative fold of topoII α protein level is expressed in comparison with that in TC71 control cells.

clear. *E1A* indirectly suppresses the *HER2/neu* promoter through the co-activator p300 (28). How this function then impacts on drug sensitivity and why the activity of certain drugs is enhanced whereas the activity of others is not are poorly defined. In this study, we show that, in addition to down-regulating HER2/neu, *E1A* transduction also significantly increases topoIIα protein expression. Using a specific human topoIIα antibody, we were able to detect a significant increase in the topoIIα protein levels in cells transduced with the *E1A* gene but not in those transduced with the *β-gal* gene. Sensitivity to VP-16 and Adriamycin has been shown to be directly linked to the amount of topoIIα protein in the cell. Indeed, we demonstrated recently that cellular resistance to VP-16 and doxorubicin could be circumvented after human topoIIα gene transfer (29). topoIIα gene transfer into MDA-VP etoposide-resistant human breast cancer cells using an adenoviral vector increased the level of topoIIα protein, and the sensitivity of these cells to VP-16 and doxorubicin was enhanced. topoIIα is the sole enzyme target of VP-16 and one of the targets of Adriamycin. In contrast, the mechanism by which cisplatin kills tumor cells has nothing to do with topoIIα. Thus, our data showing that *E1A* gene transfer enhanced the activity of VP-16 and Adriamycin, but not that of cisplatin, support our hypothesis that the mechanism by which *E1A* enhanced the chemosensitivity of Ewing's sarcoma cells was an increase in topoIIα.

Ewing's sarcoma is a relatively rare disease with limited therapeutic options. The majority of patients are initially responsive to chemotherapy with vincristine, Adriamycin, and cyclophosphamide. However, relapsed disease is usually explosive and extremely difficult to treat because of its resistance to chemotherapy. The role of oncogenes and growth factors as well as the etiology of chemoresistance in this disease is poorly understood.

We have demonstrated that HER2/neu is overexpressed in three different human Ewing's sarcoma cell lines. *E1A* down-regulated HER2/neu expression, inhibited tumor cell growth, and increased cellular sensitivity to two topoII-directed anticancer drugs. Interestingly, *E1A* gene transfer had no affect on the growth rate of normal osteoblast cells or on their susceptibility to chemotherapy (Fig. 3; other data not shown). These data indicate tumor-specific treatment effect, an important consideration for therapeutic potential. These results suggest that *E1A* gene therapy may provide a new approach for treatment of Ewing's sarcoma. Clinical trials with *E1A* gene therapy are being conducted in patients with metastatic breast cancer and ovarian cancer (30). Because there is a dearth of therapeutic options for patients with relapsed Ewing's sarcoma, our data indicate that *E1A* gene therapy may have potential in the treatment of this disease.

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