Herceptin Down-Regulates HER-2/neu and Vascular Endothelial Growth Factor Expression and Enhances Taxol-Induced Cytotoxicity of Human Ewing’s Sarcoma Cells

In vitro and In vivo

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ABSTRACT

We have previously shown that high levels of HER-2/neu protein were overexpressed in human Ewing’s sarcoma cells (TC71, SK-ES1) relative to normal human osteoblasts. The purpose of this study was to determine whether herceptin alone or in combination with chemotherapeutic agents could inhibit the growth of Ewing’s sarcoma in vitro and in vivo. Western blot analysis showed that the protein levels of HER-2/neu were decreased following herceptin treatment. Cell growth was also inhibited by herceptin in a dose-dependent manner with an IC50 of 4 mg/mL in TC71 and SK-ES1 cell lines, whereas human immunoglobulin had no effect. Northern blot and ELISA showed the RNA expression and protein levels of vascular endothelial growth factor were also inhibited by herceptin treatment with no alteration in HIF-1α protein and topoisomerase IIα expression. Furthermore, Ewing’s sarcoma tumor growth was significantly delayed by 100 mg/kg herceptin treatment in our Ewing’s sarcoma xenograft mouse model. Combining taxol with herceptin resulted in additive cytotoxicity, whereas herceptin-etoposide, doxorubicin, and 9-nitrocamptothecin combinations did not. Taxol-herceptin enhanced growth inhibition in TC71 cells in vitro compared with either agent alone. Ewing’s sarcoma growth was also delayed in vivo and mean tumor size was significantly lower in mice treated with herceptin plus taxol than in those receiving taxol or herceptin alone. These data suggest that herceptin in combination with taxol may be a therapeutic option in the treatment of Ewing’s sarcoma.

INTRODUCTION

Ewing’s sarcoma is characterized by poorly differentiated small round cells and a specific EWS-ETS chromosomal rearrangement between the EWS gene on chromosome 22 and various members of the ETS gene family (1–3). It is the second most common malignant bone tumor in children and young adults and accounts for 10% to 15% of all primary bone tumors (4). During the last two decades, patients with Ewing’s sarcoma have benefited from advances in the multidisciplinary approach to this neoplasm. Nevertheless, despite aggressive treatment, 30% to 60% of patients with localized disease and 80% of patients with metastatic disease died of the disease progression (4). The lack of new effective drugs in the treatment of sarcomas together with the side effects of high-dose regimens on young patients support the need for innovative therapeutic strategies, including treatments targeted against molecules believed to be critical for the pathogenesis and progression of Ewing’s sarcoma (5).

One such target may be the HER-2/neu gene, or erbB2 gene, which belongs to the epidermal growth factor receptor family. It encodes a 185-kDa transmembrane glycoprotein with intrinsic tyrosine kinase activity. Through its heterodimerization with other erbB receptors, HER-2/neu can mediate signal transduction from all erbB receptors and is thus involved in a variety of cell functions, including cell proliferation, differentiation, and apoptosis. HER-2/neu also plays a pivotal role in oncogenic transformation and tumorigenesis (6–10). Overexpression/amplification of HER-2/neu receptor has been identified in a variety of cancer types, including non–small cell lung cancer and carcinomas of the breast, bladder, pancreas, and ovary (11–15). HER-2/neu overexpression is associated with shortened disease-free and overall survival compared with patients who have HER-2/neu-negative tumors (16). Targeting HER-2/neu with herceptin, a humanized anti-HER-2/neu monoclonal antibody, inhibits HER-2/neu expression and blocks tumor induction by this oncogene (17). Currently, herceptin is the only Food and Drug Administration–approved antibody for treatment of cancers that overexpress HER-2/neu (17, 18). We have reported HER-2/neu overexpression in three different Ewing’s sarcoma cell lines (19). Adenovirus-E1A transfection resulted in down-regulation of HER-2/neu expression and inhibition of tumor cell growth in vitro and in vivo (19, 20). Whereas there is still controversy over whether HER-2/neu is overexpressed in patient tumors, in this report we show that herceptin inhibits not only HER-2/neu but also VEGF expression in Ewing’s sarcoma cells. Herceptin also inhibited Ewing’s sarcoma growth both in vitro and in vivo. We also show that combining herceptin with taxol, but not etoposide (VP-16), doxorubicin, or 9-nitrocamptothecin resulted in additive cytotoxicity.

MATERIALS AND METHODS

Cell Lines and Reagents. Herceptin was obtained from Genentech, Inc. (South San Francisco, CA). Taxol and
9-nitrocamptothecin were purchased from Super Gene, Inc. (San Ramon, CA). Human IgG, VP-16, and doxorubicin were purchased from Sigma-Aldrich Co. (St. Louis, MO). 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 (supplemented with 10% heat-inactivated fetal bovine serum, 1 mmol/L sodium pyruvate, 2 mM minimal essential medium vitamins, 1 mM nonessential amino acids, and 2 mmol/L glutamine) at 37°C in a humidified 5% CO2 incubator. SK-ES1 Ewing’s sarcoma cells were bought from American Type Culture Collection (Manassas, VA) and cultured in McCoy’s 5A medium with 10% fetal bovine serum. Normal human osteoblast cells were purchased from Clonetics, Inc. (San Diego, CA), and maintained in the special medium provided by Clonetics. All cells were free of Mycoplasma, as screened by Mycoplasma Plus PCR Primer Set (Stratagene, Inc., La Jolla, CA), and verified free of pathogenic murine viruses (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD). The cell lines used in in vivo experiments were from the third to the 10th passage.

**Cytostasis Assay.** Cells were seeded into 96-well cell culture plates (5,000 per well) and allowed to adhere for 5 hours before various concentrations of herceptin, taxol, VP-16, doxorubicin, 9-nitrocamptothecin, or human IgG were added; cells were cultured and treated in triplicate. The antiproliferative activity was determined 48 hours later by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (21).

**Western Blot.** Cells were placed in 100-mm dishes (2 x 10^6 per dish) 1 day before treatment, and culture medium was changed to medium containing herceptin at the IC50 concentration and cultured for another 48 hours. Cells were washed with cold PBS buffer and lysed in radioimmunoprecipitation assay buffer. Total protein (50 μg) was assayed for HER-2/neu and HIF-1α protein expression by Western blot. Densitometry analysis done for each band and expression was normalized with β-actin.

**Fig. 1** Effect of herceptin on HER-2/neu and HIF-1α protein production. TC71 or SK-ES1 Ewing’s sarcoma cells were incubated with or without herceptin at 4 mg/mL for 48 hours. The cells were then lysed in radioimmunoprecipitation assay buffer. Total protein (50 μg) was assayed for HER-2/neu and HIF-1α protein expression by Western blot. Densitometry analysis done for each band and expression was normalized with β-actin.

**Fig. 2** Herceptin inhibits the growth of TC71 and SK-ES1 cells. TC71 or SK-ES1 cells were incubated with various concentrations of herceptin or IgG (control) for 48 hours. Growth inhibition determined using the MTT assay.
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; all from Sigma Chemical Co., St. Louis, MO). The cells were then held on ice for 30 minutes before they were concentrated at 13,000 rpm for 10 minutes to get rid of cell debris. Protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). A 50-µg aliquot of total protein was boiled for 5 minutes before loading onto a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane. The specific protein bands were detected with a monoclonal anti-human HER-2/neu (Ab-3) antibody (Oncogene, San Diego, CA), anti-human HIF-1α monoclonal antibody (Novus Biologicals, Littleton, CO), anti-human p21 antibody, anti-human phospho-ERK antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or β-actin monoclonal antibody (Sigma Chemical) using the enhanced chemiluminescence Western blotting analysis system (Amersham, Piscataway, NJ) according to the manufacturers’ instructions. Densitometric analysis was done, and values were normalized against a β-actin loading control.

**Flow Cytometry Analysis.** Apoptotic cells were detected by flow cytometry as described previously (22). TC71 cells were treated as indicated for 24 hours and were washed with PBS buffer twice and fixed in 70% of ethanol overnight. We incubated 10^6 cells with RNase at 37°C for 30 minutes and stained them with propidium iodide solution (50 µg/mL propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) for 2 minutes. Cells were analyzed with a FACScan (Becton Dickinson Co., Mountain View, CA).

**Northern Blot Analysis.** Cultured cells were lysed in Trizol reagent (Life Technologies, Inc., Grand Island, NY). Total RNA was purified according to manufacturer’s instructions. A 20-µg aliquot of RNA was electrophoresed on a 1% formaldehyde/agarose gel, transferred to a Hybond-N+ membrane (Amersham Biosciences, Piscataway, NJ), and UV cross-linked at 120,000 µJ/cm² using a Spectrolinker (Spectronics Co., Westbury, NY). A human topoisomerase IIα (topo IIα) gene probe (ZII69) was a generous gift of Dr. L. Liu (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ). Human VEGF gene probe was kindly provided by Dr. B. Berse (Harvard Medical School, Boston MA). Glyceraldehyde-3-phosphate dehydrogenase probes were used as internal controls. All probes were labeled with 32P using the Rediprime DNA-labeling system (Amersham Biosciences). The membrane was hybridized at 65°C overnight and washed as previously described (23). Densitometric analysis was done using a personal densitometer SI (Molecular Dynamics, Sunnyvale, CA), and values adjusted to a glyceraldehyde-3-phosphate dehydrogenase internal control.

**Mice.** 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 maintained in an animal facility approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the U.S. Department of Agriculture, Department of Health and Human Services, and the NIH. Animals were housed for 1 week before they were used for any experiments.

**In vivo Model.** As previously described (19, 20), TC71 Ewing’s sarcoma cells in mid-log growth phase were harvested by trypsinization. Single-cell suspensions of 2 × 10^6 cells in 0.1 mL HBSS (4°C) were injected into nude mice s.c. Three to 4 days later, when tumors could be detected (1-3 mm in diameter), the tumor-bearing mice were randomly divided into several groups. In experiment 1, group 1 mice were used as nontreatment control, group 2 mice were i.p. injected with control human IgG (100 mg/kg twice weekly), group 3 and group 4 mice were injected with herceptin at 50 or 100 mg/kg on the same schedule as for group 2. In experiment 2, mice were...
divided into six groups, nontreatment or treatment with 50 mg/kg herceptin alone, 6 mg/kg taxol alone, 8 mg/kg VP-16 alone, herceptin combined with taxol and herceptin combined with VP-16. All reagents were diluted to 100 μL in PBS and given i.p., twice a week for about 3 weeks. In combination treatment groups, herceptin was given 24 hours before taxol or VP-16 was added (24). The tumors were measured every 4 days with a caliper, and their diameters were recorded. Tumor volume was calculated by the formula $a^2b / 2$, where $a$ and $b$ are the two maximum diameters. The duration of survival was recorded. When each tumor grew bigger than $2 \times 2$ cm, the mice were sacrificed, and tumor tissues were collected for analysis of HER-2/neu, VEGF, CD31 by immunohistochemical analysis, and apoptosis was quantitated by terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) assay.

**Immunohistochemical Analysis.** Histologic sections were taken from mice bearing TC71 Ewing’s sarcoma. The sections were subjected to routine pathologic analysis with H&E staining. Frozen sections fixed with acetone were incubated in 3% H$_2$O$_2$ in methanol for 10 minutes to block endogenous peroxidase and incubated in 5% normal horse serum plus 1% normal goat serum in PBS for 20 minutes to block nonspecific protein. Proliferating cell nuclear antigen staining was done with mouse anti-proliferating cell nuclear antigen antibody (Dako Co., Carpinteria, CA) as first antibody and horseradish peroxidase–labeled goat antibody against mouse IgG as the secondary antibody. Expression of the CD31 on blood vessels was detected using rat anti-mouse CD31 as the primary antibody (PharMingen, San Diego, CA) and goat anti-rat horseradish peroxidase as the secondary antibody, incubated with chromogen diaminobenzidine. The expression of HER-2/neu protein was detected by using monoclonal anti-human c-erb-2 oncoprotein (Ab-3, Oncogene, San Diego, CA) as the primary antibody and horseradish peroxidase-labeled goat antibody against mouse IgG as the secondary antibody. VEGF expression was detected by incubating the tissue sections with rabbit anti-human VEGF antibody (Santa Cruz Biotechnology) 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.

**Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling Assay.** TUNEL assay was done to detect apoptotic cells. Frozen sections were fixed with 4% methanol-free formaldehyde solution in PBS for 10 minutes and then washed thrice with PBS. The tissues were permeabilized in 20 μg/mL proteinase K solution for 10 minutes at room temperature and equilibrated in equilibration buffer for 10 minutes after which the slides were rinsed thrice with PBS. The DNA fragments were labeled with fluorescein-12-dUTP in terminal deoxynucleotidyl
transferase incubation buffer (Promega, Madison, WI) in a humidified chamber (37°C for 60 minutes) to avoid exposure to light. The reactions were terminated by transferring the slides to 2× SSC buffer [0.3 mol/L NaCl and 0.03 mol/L sodium citrate (pH 7.0)] for 15 minutes and washing them in PBS to remove unincorporated fluorescein-12-dUTP. The slides were then counterstained with 4,6 diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA) to provide a blue background. The green fluorescence of apoptotic cells can be detected with a fluorescence microscope at 520 nm.

**Ewing’s Sarcoma Patients’ Samples.** Formalin-fixed, paraffin-embedded samples from 10 Ewing’s sarcoma patients were obtained from the pathology archives at M.D. Anderson Cancer Center. The samples were evaluated for HER-2/neu and VEGF expression using immunohistochemistry.

**Statistical Analysis.** Two-tailed Student’s t test was used to statistically evaluate the differences in tumor volumes and numbers of blood vessels. P < 0.05 was considered to indicate a statistically significant difference.

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**RESULTS**

**Effect of Herceptin on HER-2/neu, Vascular Endothelial Growth Factor, HIF-1α, and Topoisomerase IIα Expression and Tumor Cell Growth In Vitro.** In an earlier study our group showed that HER-2/neu protein is overexpressed in three different Ewing’s sarcoma cell lines (TC71, SK-ES1, and A4573) compared with the level in normal human osteoblast cells (19). As shown in Fig. 1, treatment with herceptin decreased the HER-2/neu protein level in TC71 and SK-ES1 cells by 50% and 80%, respectively. Because alterations in HER-2/neu may also affect HIF-1α (25), we tested herceptin’s effect on HIF-1α expression. Herceptin treatment did not produce any alteration in HIF-1α protein (Fig. 1). Cell growth was also inhibited by herceptin in a dose-dependent manner at an IC_{50} of 4 mg/mL in both cell lines (Fig. 2).

In addition to HER-2/neu, TC71 cells expressed twice the levels of VEGF compared with normal human osteoblasts (Fig. 3A). Herceptin decreased VEGF expression in a dose-dependent manner following 48 hours of incubation. VEGF
protein production was also decreased (Fig. 3B, \( P < 0.05 \)). We have previously shown that down-regulation of HER-2/neu and VEGF by E1A results in increased expression of topoisomerase II\(\alpha\) and increased sensitivity to topoisomerase II\(\alpha\)–targeting agents such as VP-16 and Adriamycin (19). By contrast, herceptin had no statistically significant effect on the expression of topoisomerase II\(\alpha\) (Fig. 3A).

**Herceptin Reduces the Growth of Ewing’s Sarcoma**

*In vivo.* To determine whether the down-regulation of HER-2/neu and VEGF induced by herceptin had an impact on tumor growth, male athymic nude mice were inoculated with TC71 cells on one side of the flank. Three days later, when tumors measured 1 to 3 mm, mice were treated twice a week for 3 weeks with human immunoglobin (hIgG, control) or with herceptin at 50 or 100 mg/kg. An additional control group was left untreated. As shown in Fig. 4, tumor growth was delayed significantly by 100 mg/kg herceptin treatment compared with the untreated or hIgG control groups. The mean tumor volume was also significantly decreased in the mice treated with 100 mg/kg herceptin (\( P < 0.05 \)). To investigate whether this growth delay was due to reduced proliferation or possible induction of differentiation, proliferating cell nuclear antigen staining was done with tumor samples. Proliferating cell nuclear antigen–positive cells percentage were 67% and 72% in control TC71 xenograft tumors and hIgG-treated TC71 tumors, whereas proliferating cell nuclear antigen positive cell numbers were significantly decreased to 45% in tumors treated with 100 mg/kg herceptin (\( P < 0.05 \)). These support the conclusion of growth inhibition.

**Herceptin plus Taxol Reduces Tumor Growth**

*In vitro and In vivo.* We next determined the effect of combining herceptin with various chemotherapeutic agents. Herceptin did not alter cell sensitivity to 9-nitrocamptothecin, doxorubicin, or VP-16 (data not shown). By contrast, combining taxol with herceptin resulted in additive cytotoxicity as quantified by growth inhibition and apoptosis (Fig. 5). Pretreatment of TC71 cells with 3 mg/mL herceptin for 24 hours before the addition of taxol enhanced growth inhibition compared with either agent alone. These results indicate that herceptin may enhance the sensitivity of Ewing’s sarcoma to taxol. Enhanced sensitivity to taxol...
following herceptin treatment has also been shown with breast cancer cells, which overexpress HER-2/neu (24).

To investigate whether combining herceptin with chemotherapy would also enhance tumor cell killing in vivo, male athymic nude mice were inoculated with TC71 cells and then treated with herceptin followed by either taxol or VP-16. The appropriate control combination therapies were given as described in Materials and Methods. Tumor growth was delayed and mean tumor size significantly decreased in mice treated with herceptin plus taxol compared with those receiving taxol or herceptin alone (Fig. 6, *P* < 0.05). By contrast, there was no therapeutic benefit when herceptin was combined with VP-16. Survival time was also significantly increased in animals receiving herceptin combined with taxol (*P* < 0.05, data not shown).

Tumor samples were collected from each group and analyzed for HER-2/neu, VEGF, and CD31 expression (a measure of tumor vessel density) and for apoptosis. The level of HER-2/neu expression was significantly reduced in animals treated with herceptin or herceptin plus taxol (Fig. 7A3 and A5). VEGF- and CD31-positive vessels were abundant in the control and hIgG treatment groups (Fig. 7B1, B2, C1, and C2) but were decreased in the herceptin alone, taxol alone, and combination herceptin plus taxol or IgG plus taxol groups (Fig. 7B3, B4, B5, B6, C3, C4, C5, and C6). The most significant decrease was seen in the tumors treated with combination of herceptin plus taxol therapy. Apoptosis was detected using the TUNEL assay. A few TUNEL-positive cells could be seen in the herceptin or taxol treatment groups (Fig. 7D3 and D4). The herceptin plus taxol-treated tumors showed a dramatic increase in the number of TUNEL-positive cells (Fig. 7D5). These data indicate that herceptin down-regulated both HER-2/neu and VEGF expression in vivo, inhibited angiogenesis, and sensitized Ewing’s sarcoma cells to taxol-induced apoptosis.

**Effect of Herceptin on p21 Expression in Ewing’s Sarcoma Cells.** To investigate the mechanism by which herceptin enhanced the sensitivity of TC71 cells to taxol, we determined the p21 expression level before and after herceptin treatment. p21 has been shown to inhibit p34 cdc2, which is crucial for taxol-induced apoptosis. As shown in Fig. 8, p21 protein was abundant in TC71 cells as detected by Western blot. After treatment with herceptin at 100 μg/mL for 1 hour, p21 was down-regulated. This effect persisted for at least 4 hours, whereas control hIgG treatment resulted in only a small decrease in p21 at the 4-hour time point. Extracellular signal-regulated kinase (ERK) phosphorylation was also inhibited by herceptin at 4 mg/mL (Fig. 8).

**HER-2/neu and Vascular Endothelial Growth Factor Expression in Patient Samples.** Granular cytoplasmic staining was found in 4 of 10 patient tumors taken from the initial biopsy specimen. No membrane staining was detected. All 10 tumors showed significant expression of VEGF. Representative sections from five patients are shown in Fig. 9.

**DISCUSSION**

Overexpression of HER-2/neu has been correlated with enhanced tumorigenicity, enhanced metastatic potential, poor prognosis, and decreased chemosensitivity (26, 27). We have previously shown that HER-2/neu is overexpressed in three different Ewing’s sarcoma cell lines and that down-regulation of HER-2/neu following E1A gene transfer increased cellular sensitivity to VP-16 and Adriamycin but not cisplatin in vitro and in vivo. This enhanced sensitivity was secondary to the up-regulation of topoisoamerase IIα, the drug target for both VP-16 and Adriamycin (19, 20). These data indicated that manipulation of HER-2/neu may have therapeutic application in Ewing’s sarcoma. We now show that herceptin, another agent that target HER-2/neu, inhibits both HER-2/neu and VEGF expression in Ewing’s sarcoma cells and enhances their sensitivity to taxol.

Herceptin is a humanized monoclonal anti-Her2 antibody approved by the Food and Drug Administration for the treatment of cancers that overexpress HER-2/neu (17, 18). Herceptin has shown activity as a single agent and when combined with chemotherapy in the treatment of HER2-overexpressing metastatic breast cancer. The data presented here show that herceptin down-regulated HER-2/neu expression in Ewing’s sarcoma cells both in vitro and in vivo (Figs. 1 and 7A3). We have previously shown that in addition to overexpression of HER-2/neu, TC71, and SK-E1 Ewing’s sarcoma cells overexpress VEGF (28). In addition to down-regulating HER-2/neu, the data presented here showed that herceptin treatment both in vitro and in vivo lead to decreased expression of VEGF (Figs. 3 and 7B3) and decreased tumor vessel density (Fig. 7C3). We were unable, however to show any change in HIF-1α, which has been shown by others to be regulated by HER-2/neu signaling (25).

As a single agent, herceptin was only effective in suppressing tumor growth at the 100 mg/kg dose (Fig. 4). Treatment with 50 mg/kg twice weekly interperitoneally for 3 weeks resulted in minimal tumor cell apoptosis (Fig. 7D3) and no significant impact on tumor growth (Fig. 4). A recent study also showed that herceptin alone had no effect on Ewing’s sarcoma cells in vitro (29).

Alteration of HER-2/neu has been shown to impact cell sensitivity to chemotherapeutic agents. Unlike E1A gene therapy (19, 20) however, treatment with herceptin enhanced the activity of taxol but not VP-16 (Figs. 6 and 7). We presume that this is because herceptin had no effect on topoisoamerase IIα (Fig. 3), the target of VP-16. By contrast, E1A increased topoisoamerase IIα expression and protein production (19), providing a mechanistic explanation of the synergy between E1A gene therapy and VP-16 drug therapy as well as the lack of synergy seen between herceptin and VP-16.

The combination of taxol plus herceptin induced a significant increase in antitumor activity compared with single agent therapy. In addition, when the two agents were given in combination to mice with Ewing’s tumors we showed enhanced down-regulation of both HER-2/neu and VEGF, enhanced reduction in tumor vessel density, and a significant increase in tumor cell apoptosis. Herceptin or taxol administration alone had some effect, but administering herceptin 24 hours before taxol therapy augmented its activity. i.p. hIgG had no effect on tumor apoptosis (Fig. 7D2), tumor vascularity (Fig. 7C2), tumor VEGF (Fig. 7B2), or HER-2/neu expression (Fig. 7A2). Furthermore, hIgG did not enhance the efficacy of taxol (Fig. 7A6, B6, C6, and D6).
The mechanism by which herceptin enhances the activity of taxol is not clear but may involve the p21 and p34Cdc2 pathways. HER-2/neu has been shown to up-regulate p21. Inhibiting HER-2/neu down-regulates p21, which in turn allows effective p34Cdc2 activation, the essential pathway for taxol-induced apoptosis (24, 30). Indeed, we showed a decrease in p21 protein following herceptin treatment (Fig. 8).

In Ewing’s sarcoma the status of HER-2/neu in patient tumor specimens is controversial. Thomas et al. failed to show the presence of amplified HER-2/neu mRNA in archived samples from 11 Ewing’s sarcoma patients (31). Granular cytoplasmic staining but no membrane staining was found in the samples. In this study, we also showed HER-2/neu cytoplasmic staining by immunohistochemistry in 4 of 10 patient specimens. All 10 tumors showed high expression of VEGF (Fig. 9). The importance of restricted cytoplasmic staining with no membrane pattern is unclear. However, other investigators have shown a correlation with cytoplasmic staining and HER-2/neu amplification (32, 33). Furthermore, mRNA has been shown to be unstable in old fixed paraffin-embedded samples. Other investigators have examined archival Ewing’s sarcoma specimens from diagnostic biopsies by immunohistochemistry and reported no gene amplification of HER2 (29).

Clearly, a prospective, longitudinal study using fresh tissue is needed. Solely examining the initial diagnostic specimen for gene amplification may not provide sufficient information. Theoretically, relapsed disease is caused by cells that were resistant to or not eliminated by chemotherapy and/or radiation. These cells, which may make up <1% of the primary tumor, may be missed in the sampling. Therefore, the initial diagnostic biopsy may underestimate the presence of such cells. These cells may be responsible for the relapse and thus salvage therapies that target HER-2/neu even in seemingly negative staining initial biopsy tumors may indeed have clinical benefit.

Although there remains a controversy about whether or not HER-2/neu is overexpressed in Ewing’s sarcoma cells, herceptin may have therapeutic efficacy based on other mechanisms. VEGF expression was shown to be overexpressed in 17 of 31 patient samples and an independent negative predictor of survival in Ewing’s sarcoma patients (34). EWS-ETS oncoproteins were

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**Fig. 8** Effect of herceptin on p21 expression. TC71 cells were incubated with 100 μg/mL herceptin or control hIgG for 1, 2, and 4 hours or 4 mg/mL herceptin or control IgG for 1, 4, 7 hours. Total protein was extracted and quantified for p21 or phospho-ERK expression by Western blot. β-Actin used as internal control. Densitometry analysis done for each band and expression was normalized with β-actin.

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**Fig. 9** VEGF expression in patient samples. Formalin-fixed paraffin-embedded patient samples were stained with H&E (A1-A5) or by immunohistochemistry for VEGF (B1-B5).
shown to activate the VEGF promoter (34). All 10 Ewing’s tumors that we examined showed high levels of VEGF (Fig. 9). Our data showed that herceptin treatment both in vitro and in vivo led to the down-regulation of VEGF with an antiangiogenic effect (Fig. 7). Therefore, herceptin may affect VEGF expression and the vascularity of tumors that express EWS-ETS oncogenes, a hallmark characteristic of Ewing’s sarcoma. This antiangiogenic effect may in addition lead to enhanced sensitivity to chemotherapy agents such as taxol. Additionally, constitutive ERK activation has been shown not only in cells that overexpress Her2 but also in cells expressing EWS-FLI-1 chimeric proteins (35). Interfering with Her2-dependent signaling was shown to interfere with the constitutive activation of ERK-1/2 in EWS-FLI-1-expressing cells. TC-71 cells not only harbor the EWS/FLI-1 translocation, but also show constitutive activation of ERK-1/2 and MAP/ERK kinase (29). Herceptin decreased constitutive ERK and MAP/ERK kinase activation (ref. 29, Fig. 8). Therefore, treatment with herceptin may lead to the inhibition of pathways downstream of ERK activation. This in turn may affect cell sensitivity to chemotherapy. We showed decreased p21 protein following herceptin treatment (Fig. 8). p21 is downstream of the HER2-neu signaling increases the rate of hypoxia-inducible factor 1a (HIF-1a) synthesis: novel mechanism for HIF-1-mediated vascular endothelial cytoxicity by trastuzumab and chemotherapeutic agents. Clin Cancer Res 2001;7:3239–50.

In summary, we have shown that herceptin downregulated both HER-2/neu and VEGF expression in Ewing’s sarcoma cells in vitro and in vivo. Herceptin therapy led to suppression of tumor growth and enhanced the therapeutic activity of taxol. Whereas it is valid to be cautious in the use of herceptin because of the controversy over whether or not Her2 plays a role in the disease pathogenesis and because of the cardiotoxicity of the drug, one must also consider the disease prognosis and alternatives. Patients with relapsed Ewing’s sarcoma have few if any therapeutic options. Second remissions are rare and patients usually die of the disease within 1 year from the time of relapse. Based on our data and the work of others showing that modulation of epidermal growth factor receptors can interfere with EWS/FLI-1-translocated ras signaling and inhibit the mitogen-activated protein kinase pathway, we believe that judicious use of herceptin in combination with taxol in a setting of careful cardiac monitoring deserves consideration for relapsed Ewing’s patients.

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