Essential erbB Family Phosphorylation in Osteosarcoma as a Target for CI-1033 Inhibition

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Background. The role of erbB tyrosine kinases, especially Her-2, in osteosarcoma has engendered intense debate. Some investigators identified an association between low-level Her-2 expression, compared to none, and poor patient outcome. Others questioned the importance of apparent cytoplasmic expression of Her-2, since membranous overexpression is associated with poor outcome in carcinomas. We previously demonstrated that primary osteosarcoma cells express cell-surface EGFR and Her-2, with the p80 isoform of Her-4 localized to the nucleus. We wished to determine if erbB kinases in osteosarcoma were phosphorylated, and if this was required for growth. ***Procedures.*** We cultured early passage osteosarcoma cell lines in the presence or absence of the pan-erbB inhibitor CI-1033 and examined the phosphorylation status of EGFR, Her-2, and Her-4 by immunohistochemistry, cell-based ELISA, flow cytometry and two dimensional Western blot. We also assessed the impact of CI-1033 upon osteosarcoma growth and survival in vitro. ***Results.*** EGFR, Her-2, and Her-4 were constitutively phosphorylated in early passage osteosarcoma cells cultured in vitro. CI-1033 abrogated erbB receptor phosphorylation and caused growth inhibition and apoptosis in a titratable fashion with concentrations of 1 μM or more. ***Conclusions.*** EGFR, Her-2, and Her-4 are constitutively phosphorylated in early passage osteosarcoma cells in tissue culture, and erbB signaling provides essential growth and anti-apoptotic signals to osteosarcoma cells. This suggests that erbB overexpression is not required for erbB to promote malignancy, but rather that overexpression is one of several mechanisms that generate unregulated erbB signaling. Pediatr Blood Cancer 2006;46:614–623. © 2005 Wiley-Liss, Inc.

Key words: CI-1033; EGFR; ErbB; Her-2; Her-4; osteosarcoma; phosphorylation; signaling; tyrosine kinase

INTRODUCTION

Osteosarcoma is the most common bone tumor and the most common extracranial solid tumor in teenagers [1–3]. As the use of adjuvant and neoadjuvant chemotherapy has come into wide-spread acceptance over 20 years ago, the 5-year survival for this illness has risen to about 60–70% overall [1,4]. However, this plateau has not changed in more than 15 years [5]. For patients with metastatic disease, the outcomes are much worse, with less than 30% survival at 5 years [1–3,6]. Recurrent disease also has a poor outcome, with less than 20% overall survival at 5 years [7]. Chemotherapy protocols of increasing intensity have led to marginal improvements at best [8,9]. For this reason, there has been a great interest in identifying markers of therapeutic and prognostic significance.

Many markers have been discussed as having potential prognostic significance for osteosarcoma [10] including cytochrome P-450 CYP 3A4 [11], multidrug resistance protein [12,13], P53 [14–16], and Fas [16]. More recently, members of the erbB (HUGO nomenclature: ERBB) family of receptors—EGFR (ERBB1), Her-2 (ERBB2), Her-3 (ERBB3), and Her-4 (ERBB4)—have gained attention as possible prognostic and therapeutic targets in osteosarcoma [17–22]. Of these type I receptor tyrosine kinases, Her-2 has received the most attention. Increased expression of Her-2 in carcinomas, particularly breast cancer, is associated with a worse prognosis [23]. For this reason, expression of Her-2 has been examined in osteosarcoma. Five studies found an association between Her-2 expression in archival specimens of osteosarcoma and a lower overall survival [17–19,21,22], while other studies failed to confirm this observation [24–

28]. One potential reason for this discrepancy was the marked difference of Her-2 expression in immunohistochemical analysis of osteosarcoma compared to epithelial malignancies. In Her-2 overexpressing breast cancer, Her-2 immunoreactivity is restricted to the edges of the cells [23], a pattern pathologists interpret as "membranous staining." In osteosarcoma, by contrast, Her-2 expression is fainter and diffusely present throughout the entire cytoplasmic area of the cell [18,19]. In breast cancer samples, cytoplasmic expression is currently regarded as an immunohistochemical artifact. We recently have shown that such "cytoplasmic" expression for Her-2 in primary osteosarcoma cells is associated with cell surface localization of the protein [29]. In the same study we showed that two other erbB family members—epidermal growth factor receptor (EGFR)

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and Her-4—are also widely expressed in osteosarcoma samples.

The expression of erbB family members in osteosarcoma allows the possibility that these markers can be therapeutic targets. Multiple drugs recently have been developed that act on one or more members of the erbB family. Two available monoclonal antibodies, Trastuzumab [30] and Cetuximab [31,32], which recognize Her-2 and EGFR respectively, have a beneficial therapeutic effect against erbB overexpressing carcinomas. In addition, several small molecule inhibitors have been developed to block signaling by these receptors [33–40]. Some of these agents are now FDA-approved while others are in late clinical trials. If an essential role for these receptors can be shown in osteosarcoma, then patients may benefit from therapy targeting these receptors.

ErbB family member signaling has been studied in some detail in epithelial malignancies. Within the cytoplasmic domain of EGFR, phosphorylation of particular cytosolic tyrosine residues provides docking sites for, and activation of, particular second messengers. These include tyrosine 845 for src [41], tyrosine 992 for phospholipase Cγ [42], and tyrosine 1068 for Grb-2 [43,44]. In addition, phosphorylation of tyrosine residue 974 has been shown to induce trafficking of activated EGFR receptors into clathrin-encoded pits [45], from which the activated receptor continues to signal for up to an hour. Phosphorylation of tyrosine residue 1045 leads to binding of Cbl, which causes receptor ubiquination and degradation [45]. The particular second messengers triggered by erbB activation depend in part upon the density of those second messengers in a given cell. Similar residues have been identified for src (tyrosine 877) [46] and Grb-2 (tyrosine 1248) [47] within Her-2's cytoplasmic domain. Her-4 signaling is less well characterized.

Here, we sought to determine if these critical tyrosine residues of the erbB family were phosphorylated within early passage osteosarcoma cell lines, and if this phosphorylation were important to the biology of osteosarcoma. To assist in this analysis, we utilized CI-1033, a small molecule inhibitor specific for all members of the erbB family which has been shown to cause loss of erbB phosphorylation in carcinomas [48]. This compound inhibits erbB molecules in the low nanomolar range in cell-free systems and retains specificity through at least 20 μM concentration in whole cell/culture systems [49]. No other targets for CI-1033 have been described [49]. We reasoned that CI-1033-mediated dephosphorylation of erbB family members would facilitate identification of constitutive phosphorylation in cultured cells, especially in lines where the receptors are present in low abundance. The same panel of early passage osteosarcoma cell line described in our previous work was examined for phosphorylation of specific tyrosine residues in the presence or absence of CI-1033 using cell based ELISA and flow cytometry. We also examined two dimensional (2D) gel electropheresis to see if an alteration of isoelectric point (pI, the mobility in a pH gradient) would indicate

the phosphorylation state of erbB receptors. Finally, we assessed the impact of erbB receptor blockade with the small molecule inhibitor CI-1033 on osteosarcoma cell growth in vitro and assessed whether this blockade would induce apoptosis using flow cytometry for annexin-V and 7 aminoactinomycin-D (7-AAD) staining. Collectively these studies demonstrate the extent of tyrosine phosphorylation in early passage osteosarcoma cell lines cultured in vitro and the impact of receptor blockade on erbB phosphorylation for osteosarcoma cells and cell growth.

METHODS

Cell Lines

All human tumor cell lines were obtained/derived with the approval of the Institutional Review Board of the University of Michigan Medical Center and have been described previously [29]. Cells were cultured in DMEM supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 mcg/ml), and L-glutamine (292 mcg/ml, Gibco/Invitrogen, Grand Island, NY) and 1% insulin/transferrin/selenium (Gibco). Primary osteosarcoma cell lines WOL and COL were derived from patients treated at the University of Michigan and were analyzed at fewer than 20 passages. Line OS-187 was the kind gift of Dr. Richard Gorlick, Memorial Sloan Kettering Cancer Center, New York. The stable cell lines Saos-2 [50] and SJSA [51] are available from ATCC as are control lines SKOV-3 [52] and MCF-7 [53].

Immunohistochemistry

Five micron sections of paraffin-embedded archival tumor specimens were assessed for expression of erbB family members as described previously [29]. The following antibodies were used: anti-EGFR (clone H11, Dako, 1:100), anti-Her-2 (Rabbit polyclonal antibody, cat# A0485 DAKO, 1:200), or anti-Her-4 (clone HFR-1, Neomarkers, Fremont, CA, 1:200). Digital photomicrographs were obtained at 400× magnification.

Cell-Based ELISA

Cell-based ELISA assessment of protein expression and phosphorylation was performed as described [54]. Briefly, primary osteosarcoma cell lines were seeded in 96 tissue culture plates at a density of 2×10^4 cells/well and cultured overnight. The following day CI-1033 was added to give the desired final concentrations. Controls included cells cultured without CI-1033 and carcinoma lines known to respond to CI-1033. After 14 hr of drug exposure the cells were washed with PBS, fixed with 4% formaldehyde in PBS and permeablyzed with 0.1% Triton-X-100 in PBS. After blocking with 10% BSA, the antigens were detected with primary rabbit antisera and a secondary goat anti-rabbit

antibody conjugated to horseradish peroxidase. All antibodies were obtained from Cell Signaling Technologies (Beverly, MA). Detection was performed with ABTS solution (Roche). Antigen presence was quantitated by optical density measurements taken at a wavelength of 405 λ less the signal measured from cells stained with the secondary antibody only. Significance was determined by Student's t test using the GBStat data analysis software (Dynamic Microsystems, Inc., Silver Spring, MD).

Flow Cytometry

Primary osteosarcoma cells were grown to approximately 75% confluence and then exposed to CI-1033. After 4 hr of drug exposure, cells were mobilized from the dishes using a non-enzymatic cell dissociation buffer (Invitrogen). To directly assess EGFR phosphorylation, cells were fixed with 4% paraformaldehyde, then permeablyzed with 0.1% Triton-X-100 in PBS. The cells were stained with a primary rabbit Ab specific for EGFR phosphorylated at tyrosine residue 1068 (Rabbit polyclonal # 2234, Cell Signaling) and a phycoerytherin-conjugated secondary goat-anti-rabbit Ab, and analyzed on an EPICS flow cytometer using XL System II software (Beckman-Coulter). Because commercially available receptor-specific antiphosphotyrosine antibodies do not routinely work in flow cytometry, only selected phosphotyrosine residues were assessed using this technique.

For direct assessment of EGFR density at the plasma membrane, unfixed osteosarcoma cells were stained with an anti-EGFR antibody directly coupled to PE (clone EGFR-1, Becton Dickinson, San Jose, CA). Immunoconjugated beads (Quantum Simply Cellular, Bangs Laboratories, Fishers, Indiana) were stained in parallel to allow quantitation of cell surface EGR expression using methods that we described previously [29].

To measure apoptosis by flow cytometry, cells were treated with CI-1033 for 4 hr, then single-cell suspensions were made as described above. Cells were stained with Annexin-V conjugated to FITC (BD-Pharmingen), and with 7-amino-actinomycin D (7-AAD) (BD-Pharmingen) according to the manufacturer's directions. All analysis was performed using an EPICS flow cytometer.

2D Gel Electrophoresis

Primary osteosarcoma cells were grown in 150 mm plates to ∼75% confluence prior to the addition of CI-1033. Fresh media was added with either 5 µM CI-1033 or no drug and the cells cultured overnight (∼15 hr). The following morning, whole cell lysates were prepared by standard techniques [55]. Lysates were separated first by isoelectric focusing on a pH gradient from 4 to 10 and then by SDS–PAGE using a 4 to 20% acrylamide gel, and the proteins transferred to PVDF membranes. The membranes were probed for Her-4 (rabbit polyclonal sc-283, Santa Cruz, 1:500) and antigen was

detected by chemiluminescence according to the manufacturer's directions (Amersham Biosciences, Piscataway, NJ).

Growth Inhibition Assay

Primary osteosarcoma cells were seeded in 6 well plates at a density of 10,000 cells per well and grown in the presence of defined concentrations of CI-1033 (1, 2, 3, 5, 7.5, or 10 µM) or no drug. Parallel wells were seeded to allow triplicate samples to be counted each day, and media and drug were refreshed daily. Cell growth was quantified using a Coulter Counter as described [56]. Statistical analysis was performed using Student's independent t-test comparing cell yield for each drug concentration to that of the untreated cells, using Statistica Software (StatSoft, Inc., Tulsa, OK).

RESULTS

EGFR, Her-2, and Her-4 Expression in Osteosarcoma

The immunoreactivity observed for the erbB family in immunohistochemical analysis of osteosarcoma has been a source of debate and confusion. To demonstrate the expression of erbB family members in osteosarcoma, archival osteosarcoma specimens were sectioned and stained with standard immunohistochemistry techniques for EGFR, Her-2, and Her-4 expression. Characteristic staining, performed on a pretreatment archival biopsy specimen, is shown for illustrative purposes (Fig. 1). EGFR showed diffuse antigen reactivity throughout each cell. Focal cells demonstrated Her-2 staining diffusely present throughout the cell with no evidence of membranous immunoreactivity for either EGFR or Her-2. Her-4 expression was seen in two patterns: cells that have diffuse cytoplasmic immunoreactivity, and cells in which the immunoreactivity was localized predominantly within the nucleus. This staining is similar to that found by others [17,18] and to that described in our previous report [29].

Constitutive erbB Phosphorylation Elimination by CI-1033

Cell-based ELISA. Our previous studies have shown that the antigen reactivity such as that seen in Figure 1 is due to the cell surface expression and internal expression of full length EGFR and Her-2 by primary osteosarcoma lines and by expression of an 80 kDa fragment of Her-4 in primary osteosarcoma cell lines [29]. It is vital to know if these receptors are present in osteosarcoma in a phosphorylated state, and thereby associated with activation of second messenger signaling. The erbB receptor density is much lower in primary osteosarcoma cell lines than that seen in epithelial malignancies, making traditional techniques for assessing protein phosphorylation, such as Western blot, more difficult.
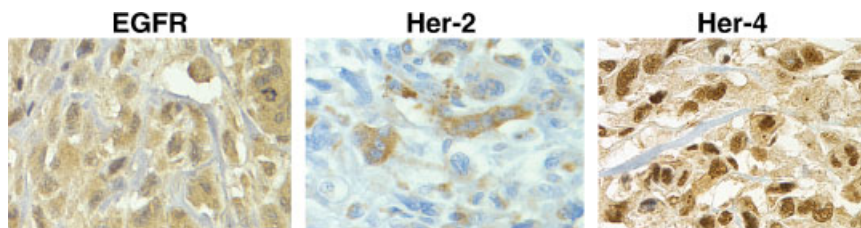
Fig. 1. erbB family member expression in archival osteosarcoma biopsy specimen. Formalin-fixed, paraffin-embedded, pre-treatment biopsy specimen of osteosarcoma was assessed for expression of EGFR, Her-2, and Her-4 by immunohistochemistry, visualized by DAB (brown). Photomicrographs were obtained at 400× magnification. Figure demonstrates the characteristic staining pattern for the three antigens.

To assess erbB phosphorylation in osteosarcoma we utilized a more sensitive technique [54]—a cell based ELISA (Fig. 2). To provide further information about the phosphorylation state of the receptors we also used a small molecule inhibitor of erbB family signaling: CI-1033. This compound binds irreversibly to the kinase domain of all erbB family members. Once CI-1033 is bound to the receptor it is no longer able to participate in kinase reactions and becomes dephosphorylated. To assess for erbB phosphorylation, osteosarcoma cell lines were cultured and analyzed without the addition of exogenous ligand, though some EGF or other erbB ligands may be present in the fetal calf serum used in there experiments. Overnight treatment with CI-1033 caused only a small change to EGFR or Her-2 expression in primary osteosarcoma cells. At CI-1033 concentrations of 5 µM or higher, cell death, decreasing the number of cells stained and therefore the total amount of EGFR and Her-2 per well, accounts for a loss in measurable EGFR and Her-2 protein. However, all concentrations of CI-1033 induced a significant decrease in measurable phosphorylation at tyrosine residues 845 and 1068 of EGFR, responsible for Src [57] and Ras/MAPK [43] signaling respectively. The corresponding residues of Her-2, tyrosines 877 and 1248 [47] were significantly dephosphorylated by CI-1033 at a concentration of 3 µM or higher. Similar results to those shown in Figure 2 for OS 187 were obtained from cell lines COL, WOL, SJSA, and Saos-2.

Flow cytometry. To visualize the presence of phosphorylated tyrosine residues within the erbB receptors expressed in osteosarcoma cells, we utilized flow cytometry (Fig. 3). In this experiment, osteosarcoma cells are fixed with formaldehyde and the plasma membrane permeablized with detergent to allow staining of the cytoplasmic tail of EGFR. There was a shift to the right seen for florescence intensity of cells grown under normal conditions and stained for EGFR phosphorylated at residue 1068 compared to cells stained with the second layer only. Exposure to CI-1033 completely eliminated this increase, indicating receptor dephosphorylation by the drug.

EGFR internalization is blocked by CI-1033. Previous studies have shown that phosphorylation at tyrosine residue 974 of EGFR is responsible for binding of AP-2 [58] which then leads to internalization of activated EGFR

receptors within clathrin-coated pits [45]. We reasoned that, if EGFR tyrosine residue 974 were constitutively phosphorylated in osteosarcoma cells grown in tissue culture, then much of the total cellular EGFR would be internalized and not amenable to staining for flow cytometry without permablizing the plasma membrane. To assess the phosphorylation state of EGFR tyrosine residue 974 we chose to
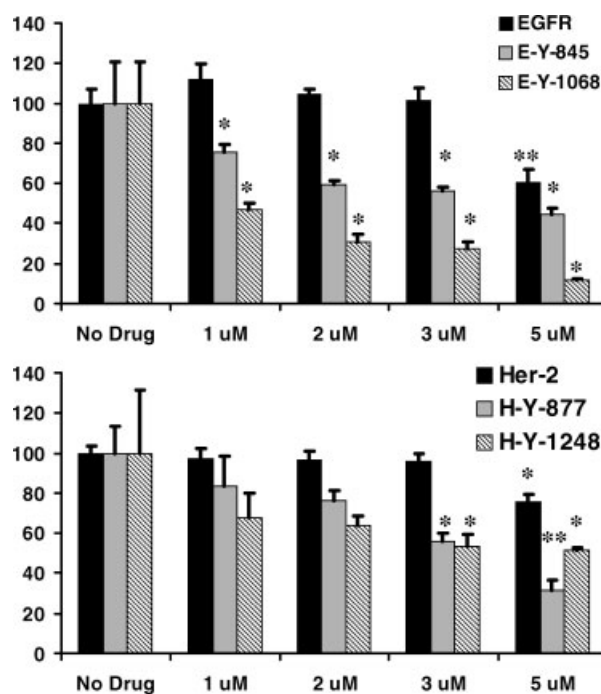

Fig. 2. Cell-based ELISA assessment of phosphorylation of EGFR and Her-2 inhibited by CI-1033. OS 187 cells were seeded in triplicate in 96 well plates, cultured for 24 hr, then treated with 1, 2, 3, or 5 µM CI-1033 or no drug overnight, then assessed for total EGFR or Her-2 or for phosphorylation of these receptors at specific tyrosine residues. Black bars represent total receptor protein (EGFR in the upper panel and Her-2 in the lower panel). Light gray bars represent phosphorylation of the src binding site for each receptor, and dark gray bars represent phosphorylation of the Grb-2 binding site. Graphs depict change in optical density with drug treatment compared to untreated cells (100%). Comparisons were made between each drug dose and untreated cells, and significance assessed with Student's t test. A single asterisk indicates $P = 0.05$, and a double asterisk indicates $P = 0.01$. A representative experiment of four is shown. Similar results were obtained with osteosarcoma cell lines COL, WOL, SJSA, and Saos-2.
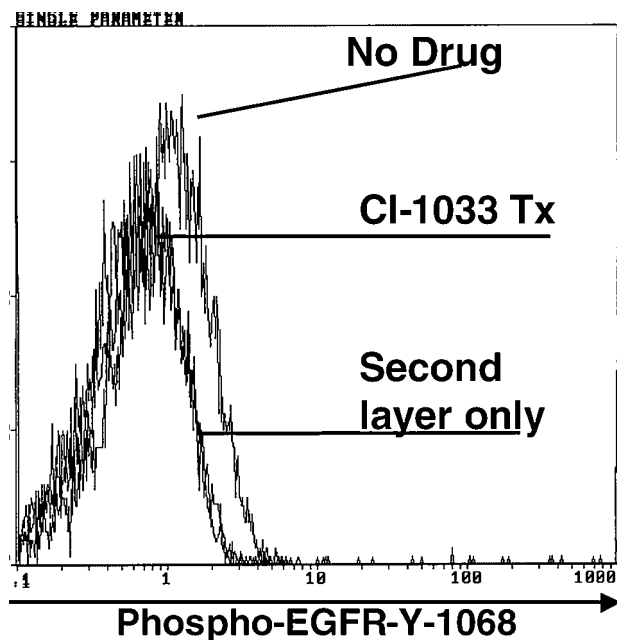
Fig. 3. Flow cytometry assessment of CI-1033 impact upon EGFR tyrosine residue 1068 phosphorylation. Single cell suspensions of OS 187 cells that had been treated for 4 hr with 5 μM CI-1033 or no drug were fixed, permeablized, and stained with a monoclonal antibody specific for the cytoplasmic domain of EGFR phosphorylated at tyrosine residue 1068. Single color overlay histograms compare EGFR-Y-1068 expression detected with anti-Ig conjugated to PE to the signal from cells stained with the secondary antibody alone. Similar results were obtained with osteosarcoma cell lines COL, WOL, and Saos-2.

Fig. 4. Flow cytometry assessment of CI-1033 impact upon cell-surface expression of EGFR. Single cell suspensions of cultured OS 187 cells or A431 control cells that had been treated for 4 hr with 5 μM CI-1033 or no drug were analyzed with anti-EGFR MAb directly conjugated to PE or with isotype controls. Single color overlay histograms are shown. EGFR expression quantified by comparison to standardized immunoconjugated beads is displayed in thousands of molecules per cell for untreated and CI-1033-treated osteosarcoma cells. Similar results were obtained with osteosarcoma cell lines WOL and COL.

examine by flow cytometry total cell surface EGFR expression on osteosarcoma cells that were grown in the absence or presence of CI-1033 (Fig. 4). As with our previous reports, OS-187 expressed about 25,000 cell surface EGFR receptors under normal conditions. After exposure to CI-1033 for 4 hr cell surface EGFR receptor expression increased to about 85,000 receptors. Similar findings were made using osteosarcoma lines COL and WOL. This increase in receptor density is not observed when EGFR is present in the dephosphorylated state: the epitheloid carcinoma line A-431, where the bulk of the EGFR receptors are known to be in the dephosphorylated state [59], does not show an upregulation upon exposure to CI-1033 (Fig. 4).

Two-dimensional gel electrophoresis of Her-4. Because commercial antibodies that recognize specific phosphorylation sites within Her-4 cytoplasmic domain are not available, the techniques used in Figures 2 and 3 could not be used to assess the phosphorylation state of Her-4 in osteosarcoma cells. The phosphorylation state of Her-4 was assessed using 2D-gel electrophoresis (Fig. 5). Lysates from early passage osteosarcoma cell lines COL and WOL treated with no drug showed a range of pI consistent with varied phosphorylation of the multiple potential phosphorylation sites within the cytoplasmic domain of the receptor. Only the p80 isoform of Her-4 is seen in great density with this
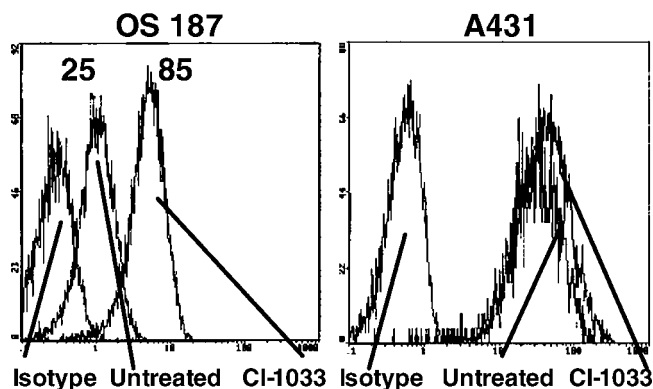
technique. Upon exposure to CI-1033 a Her-4 species with a much higher pI is observed. The development of this spot is consistent with the dephosphorylation of the 80 kDa isoform of Her-4. We also see the development of a 60 kDa fragment that also stains for Her-4. The etiology of this fragment is not known.

Effect of erbB Dephosphorylation by CI-1033 on Osteosarcoma Growth and Survival

Growth inhibition assay. To determine if dephosphorylation of erbB receptors in osteosarcoma impacts cell growth, early passage osteosarcoma cell lines were grown in the presence of defined concentrations of CI-1033 for a period of up to 4 days, and the cell yield from individual wells was quantified daily. The average yield from triplicate wells was compared to the growth of cells exposed to no drug (Table I). Both media and drug were changed daily. We saw a significant decrease in the rate of tumor cell growth with 1 μM CI-1033, with an estimated IC^{50} for CI-1033 of slightly higher than 1 μM for both OS 187 and COL. Three μM or greater concentration of drug led to complete growth inhibition. Treatment with CI-1033 at concentrations of 7.5 or higher led to rapid cell loss from the culture, with few cells recovered after a single day (data not shown). The inhibition of cell growth was not dependent on Her-2 expression since similar amounts of growth inhibition were observed in the Her-2 expressing OS-187 cell line and in the Her-2 negative COL line. Similar results were obtained with early passage osteosarcoma lines WOL, JOL, KOL, and MAOS, the established cell line Saos-2 and the control ovarian cancer cell line SKOV-3 (data not shown).
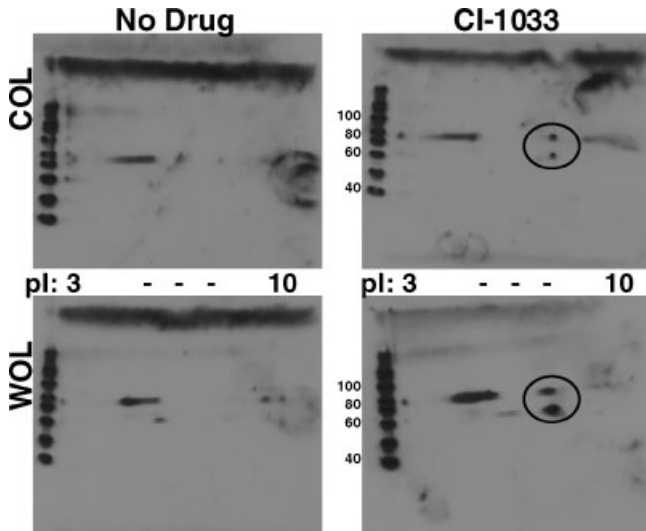
Fig. 5. 2D Western analysis of CI-1033 impact upon Her-4 phosphorylation. Whole cell lysates of WOL and COL cells treated overnight with 5 μM CI-1033 or no drug were separated by isoelectric focusing on a pH gradient (pI), then by size via SDS–PAGE, and proteins transferred to PVDF membranes. Her-4 protein was identified with monoclonal antibodies specific for the cytoplasmic domain of the protein. A circle identifies the 80 and 60 kDa species of higher pI that arise with exposure to CI-1033.

Apoptosis assay. We next determined if growth inhibition observed in cells cultured with CI-1033 was due to the induction of apoptosis. To address this question early passage osteosarcoma cell lines were treated for 4 hr with defined concentrations of CI-1033 or no drug, then assessed for apoptosis by flow cytometry for annexin V and 7-AAD (Fig. 6). Controls for apoptosis were obtained by exposing cells to etoposide (not shown). Exposure to CI-1033 increased the rate of apoptosis in primary osteosarcoma cells in a titratable fashion. At baseline OS-187 had approximately 20% of cells undergoing apoptosis. By

contrast after exposure to 1 μM CI-1033, 50% of the recoverable cells are undergoing apoptosis, and 80% with 5 μM CI-1033. COL was more resistant to programmed cell death in a 4 hr assay, with 18% of cells undergoing apoptosis at baseline, increasing to 21% with 1 μM CI-1033 and 69% with 5 μM CI-1033.

DISCUSSION

Investigations of the erbB family in osteosarcoma in recent years have focused exclusively on one family member—Her-2. The debate over the role of Her-2 in osteosarcoma pathogenesis has involved great controversy, with groups arguing for and against the hypothesis that Her-2 expression confers a worse prognosis [17–19,24–28]. The resolution of this controversy requires four points: first, the expression of Her-2 and the other erbB family members needs to be delineated. Second, there must be an assessment of the activation state of the erbB family of receptors. Third, it is important to measure the impact of erbB signaling upon cell growth. Finally correlations must be made between receptor expression, receptor activation status and disease outcome.

For Her-2 expression, this process began with the final step. Five different groups found that Her-2 expression did, in fact, correlate with a worse outcome [17–19,21,22]. The typical pattern for Her-2 in osteosarcoma is that seen in Figure 1: diffuse antigen reactivity throughout the cytoplasm of positive cells. Our previous work helped define the receptor expression for the erbB family in osteosarcoma [29]. In that study, we showed that both EGFR and Her-2 localize, at least in part, to the cell surface in primary osteosarcoma cells. We also showed that Her-4 was present in predominantly the 80 kDa form. This isoform of Her-4 is generated through cleavage of the full-length molecule by ADAM-17 (also called TACE) and γ-secretase [60–62], a process that is

TABLE I. Inhibition of Osteosarcoma Cell Growth by CI-1033

	Day 1			Day 2			Day 3			Day 4		
	Cell #	SD	P	Cell #	SD	P	Cell #	SD	P	Cell #	SD	P
OS187												
Control	21,700	936		57,300	1,032		151,000	13,264		309,900	23,853	
1 μM	16,600	977	**0.0028**	41,600	6,323	1.0132	81,800	3,193	**0.0009**	169,900	19,184	**0.0013**
3 μM	11,500	64	**<0.0001**	19,600	4,019	**<0.0001**	16,900	2,532	**<0.0001**	15,700	2,239	**<0.0001**
5 μM	5,100	980	**<0.0001**	9,900	1,306	**<0.0001**	10,300	405	**<0.0001**	7,200	605	**<0.0001**
COL												
Control	5,200	1,721		13,200	1,551		25,700	1,942		78,800	6,281	
1 μM	7,000	906	0.1732	10,800	4,746	0.4483	13,600	5,832	**0.0274**	58,432	5,285	**0.0126**
3 μM	2,600	688	0.0758	2,200	392	**0.0003**	4,200	324	**<0.0001**	3,900	1,104	**<0.0001**
5 μM	2,600	545	0.0694	1,300	382	**0.0002**	1,400	271	**<0.0001**	1,091	64	**<0.0001**

Early passage osteosarcoma cell lines (OS 187 and COL) were grown in the presence of defined concentrations of CI-1033 or no drug for four days. The average cell yields and standard deviations are presented. Comparisons between each drug treatment and the control (no drug) cells from the same day with a P value <0.05 are identified with bold, underlined text. Results of representative experiments from two cell lines are shown. Similar results were obtained from early passage lines WOL, JOL, KOL, and MAOS, from the established osteosarcoma line Saos2, and from the control cell line SKOV-3.
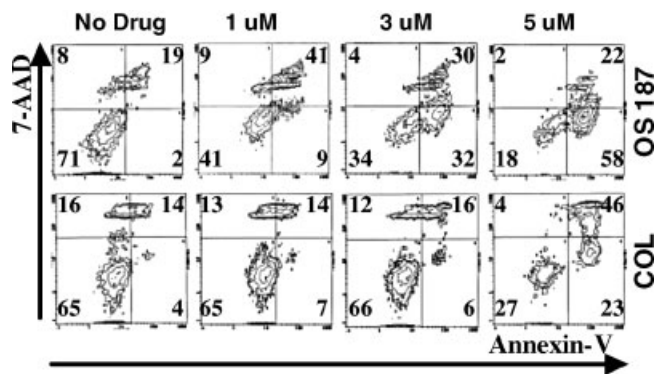
Fig. 6. Induction of osteosarcoma apoptosis by CI-1033. Single cell suspensions of OS 187 and COL cells that had been treated for 4 hr with 0, 1, 3, or 5 μM CI-1033 in DMSO were stained with annexin V and 7-AAD and assessed by flow cytometry. Cells that stained for neither marker were living, viable cells. Cells that were positive for 7-AAD only had disrupted cellular membranes without loss of membrane polarity and were either necrotic or were damaged in mobilization from the tissue culture dish. Cells that were positive for annexin V were in early apoptosis, and cells positive for both reagents were in late apoptosis. Thus the percentage of cells undergoing apoptosis is the sum of the right upper and right lower quadrants. The percentage of cells in each quadrant is shown for each histogram.

believed to require prior activation of the receptor. In the present work, we show the activation state of erbB receptors in osteosarcoma and the impact of erbB phosphorylation on osteosarcoma growth.

Immunoprecipitation of erbB family members is difficult in cells that do not overexpress the proteins, i.e., in cells having less than 1 to 2 million molecules per cell. For this reason, our group used more sensitive but less familiar methods such as cell based ELISA, direct flow cytometric assessment, and 2D gel electrophoresis. These techniques provided evidence for the phosphorylation of EGFR, Her-2, and Her-4 in early passage osteosarcoma cell lines grown in 10% fetal calf serum without exposure to additional exogenous ligand. By contrast, the typical overexpressing EGFR line, A431, must have exposure to EGF or another EGFR ligand for the receptor to be phosphorylated [59].

The assessment of phosphorylation for EGFR, Her-2, and Her-4 was facilitated by the loss of this phosphorylation in cells treated with the pan erbB inhibitor CI-1033. In all of the techniques utilized—cell ELISA, flow cytometry, and 2D gel electrophoresis—CI-1033 induced the loss of phosphorylation at specific phosphotyrosine residues or in total receptors compared to the expression seen in primary osteosarcoma cells grown in media alone.

Flow cytometric analysis of cells exposed to CI-1033 also confirmed an important aspect of receptor trafficking for EGFR. In our previous report, we suggested that much of the EGFR in primary osteosarcoma cells appeared to be localized to an internal or non-cell surface location [29]. This receptor internalization is not surprising, since previous studies have shown that EGFR phosphorylated at tyrosine

residue 974 is rapidly internalized—occasionally as quickly as 5 min [45]. It has also been shown that after internalization EGFR can continue to transmit second messenger signals for as much as an hour prior to being brought into lysosomes and degraded [45]. Further, Her-2 is the preferred dimerization partner for both EGFR and Her-4 [63,64], and when Her-2 and EGFR are expressed in a cell at roughly equal densities, phosphorylation of EGFR results in the trafficking of Her-2 into a cytosolic location: clathrin coated vesicles [65]. Thus a cytoplasmic location of either EGFR or Her-2, as seen in archival tumor samples such as that shown in Figure 1 and in our previous report [29], could be interpreted as a sign of receptor activation.

The data presented here confirm that EGFR in osteosarcoma cells grown in serum-containing media is not only constitutively phosphorylated, but localized predominantly to an intracellular compartment. Since the control treated osteosarcoma cells express about 25,000 EGFR receptors on their cell surface and CI-1033 treated cells express more than three times that number at the cell surface, this dramatic upregulation of receptor expression is evidence of the activated state of EGFR in osteosarcoma. Specifically, this upregulation after CI-1033 treatment is evidence, in cells grown in these conditions, of constitutive phosphorylation at tyrosine residue 974, since it is this residue that provides the docking site for AP-2, which mediates trafficking to coated pits [45].

It is not clear how the erbB family members in osteosarcoma become constitutively phosphorylated. While fetal calf serum may be providing erbB family ligands in our experiments, the immunoreactivity of erbB antigens in archival osteosarcoma samples is also consistent with receptor phosphorylation and active signaling [29]. One suggested mechanism for erbB activation has been the secretion of EGF or other ligands by osteosarcoma cells, leading to phosphorylation and stimulation in an autocrine or paracrine fashion [14]. Another mechanism could be phosphorylation of erbB receptors without ligand by activated Src molecules. Further, it is possible that other receptor tyrosine kinases such as platelet derived growth factor receptor α or β or c-kit might in turn activate the erbB receptors in some cell lines. Finally it is possible for mutations of the erbB receptors themselves to lead to constitutive activation. This mechanism has been shown for EGFR in glioblastoma multiforme where the expression of a truncated, constitutively active isoform—EGFRvIII—is associated with a worse outcome and unregulated activation of erbB signaling pathways [66–69].

What may be more important for osteosarcoma biology than the mechanism of erbB activation is the result of that phosphorylation: that when erbB signaling is impeded by a small molecule, osteosarcoma cells enter growth arrest and undergo apoptosis. Multiple mechanisms of erbB activation may be used by different tumors types or by similar tumors in different individual patients. It is possible, or indeed likely,

that a common second messenger pathway, induced by unregulated signaling from the erbB family, promotes the malignant phenotype in osteosarcoma. The signaling of the erbB family is complex with no fewer than six different second messenger systems being involved in regulation and signal transduction. It is unclear at present which of these pathways is most important for promoting the malignant phenotype in primary osteosarcoma. This subject merits further investigation.

ErbB signaling in osteosarcoma may provide an important therapeutic target. Several drugs have recently been licensed for clinical use that specifically target one or more members of this family. These include monoclonal antibodies such as Trastuzumab [30] and Cetuximab [31,32], which target Her-2 and EGFR respectively, and the small molecule inhibitor Gefitinib [36,70,71]. CI-1033, the pan-erbB inhibitor used in our studies, is in phase II clinical trials. The in vitro data about the impact of erbB inhibition on osteosarcoma growth shown here are encouraging. It may be that treatment with drugs that target the erbB family, particularly small molecules that are not affected by receptor internalization, will provide a new therapeutic option for this disease that has shown no significant improvement in nearly twenty years.

The data presented here also provide new insights into how erbB receptor expression can affect disease severity. The reigning paradigm, dominant in the breast cancer literature and influencing the interpretation of many other studies, suggests that overexpression of one or more erbB family members is the primary mechanism by which these molecules promote a worse disease phenotype. Perhaps overexpression in and of itself is less important than the activation state of the receptors. Here we provide evidence that at least one of the erbB receptors seems to drive the growth of early passage osteosarcoma cell lines in vitro, such that inhibition of this signaling causes apoptosis. This demonstrates that one does not need overexpression of these receptors for their presence to affect cell growth, at least in vitro. The several reports showing an association between erbB expression in archival osteosarcoma specimens and reduced survival [17–20] also suggest that overexpression is not required for erbB signaling to impact the natural history of the disease in patients, either. We would suggest that overexpression is just one of several mechanisms that can induce the condition that does promote the more severe phenotype, which is uncontrolled erbB signaling. Important questions remain regarding the role of erbB expression in osteosarcoma. A key question is, which signaling pathways are of greatest importance in osteosarcoma, and how do these second messenger signals promote the malignant phenotype? The answers to these questions will help to provide insights that will guide future treatment.

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