

# Sensitization of breast cancer cells to radiation by trastuzumab

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## Abstract

HER2, a member of the human epidermal growth factor (EGF) receptor family, not only plays important roles in the progression of breast cancer tumorigenesis and metastasis, but may protect cancer cells from conventional cytotoxic therapies as well. In the current study, we evaluated the effect of targeting HER2 on radiosensitization of human breast cancer cells. Using six breast cancer cell lines with various levels of HER2 (BT474, SKBR3, MDA453, MCF7, ZR75B, and MDA468), we found that trastuzumab (Herceptin), a humanized monoclonal antibody that may inhibit breast cancer cell proliferation but does not induce apoptosis when used alone, enhanced radiation-induced apoptosis of the cells in a HER2 level-dependent manner. We furthered this study in MCF7 cells transfected for high levels of HER2 (MCF7HER2). Compared with parental or control vector-transfected MCF7 cells, MCF7HER2 cells showed increased phosphorylation of at least two important HER2 downstream molecules, protein kinase B/Akt and mitogen-activated protein kinase (MAPK), and increased resistance to radiotherapy, as shown by reduced induction of apoptosis and increased cell clonogenic survival after radiation. Exposure of the cells to trastuzumab down-regulated the levels of HER2 and reduced phosphorylation levels of Akt and MAPK in MCF7HER2 cells, and sensitized these cells to radiotherapy. When specific inhibitors of the phosphatidylinositol 3-kinase (PI3-K) and MAPK kinase (MEK) pathways were used, we found that exposure of MCF7HER2 cells to the PI3-K inhibitor LY294002 inhibited Akt phosphorylation and radiosensitized the cells, whereas the radiosensitization effect by the MEK inhibitor PD98059 was relatively weaker, albeit the phosphorylation of MAPK was reduced

by PD98059 treatment. Our results indicate that the PI3-K pathway might be the major pathway for trastuzumab-mediated radiosensitization of breast cancer cells. (*Mol Cancer Ther.* 2003;2:1113–1120)

## Introduction

Radiotherapy of patients with breast cancer plays an essential role in local control of the disease. Randomized trials have demonstrated the efficacy of radiation therapy in the treatment of breast cancer (1–4). Reduction in the risk of recurrence has been shown in patients with breast conservation for ductal carcinoma *in situ* and in patients with invasive cancers after breast lumpectomy and conservation (3). Recent results from chemoradiation studies further confirmed added benefits when radiotherapy is combined with chemotherapy, in terms of both better local control and improved survival (5, 6). Thus, the role of radiotherapy in the management of breast cancer is well established.

Breast cancer cells are relatively refractory to ionizing radiation-induced DNA damage and apoptosis (7). While many inherent factors may modulate cellular response to DNA damage, such as the p53 status of the cell, level and activity of the Bcl-2 family, activity of NF- $\kappa$ B, the cell survival signals resulting from growth factor receptor-mediated signals, and the cell death signals following activation of death receptor pathways (7, 8), a recent study explored the potential association between several known molecular markers and pathological response from the original tumors following a regimen of preoperative concurrent treatment with paclitaxel and radiation, and found that only HER2 and estrogen receptor seemed to be significantly associated with the extent of pathological response to the regimen, that is, tumors with low levels of HER2 and negative estrogen receptors were more likely to respond to the regimen (9).

HER2, a member of the human epidermal growth factor (EGF) receptor family, encodes a  $M_r$  185,000 transmembrane glycoprotein receptor protein that has intrinsic tyrosine kinase activity (10). HER2 is overexpressed in approximately 30% of human breast cancers (11) and plays important roles in the progression of breast cancer tumorigenesis and metastasis (12). Patients whose tumors overexpress HER2 have an overall poorer prognosis and decreased survival compared with patients with HER2-negative tumors (13). In contrast to other members of the EGF receptor family, HER2 remains an orphan receptor, with no soluble ligand identified so far, but the receptor plays a pivotal role in the HER network, being the preferred and most potent heterodimerization partner for other HER members (14, 15). Activation of HER2 that results from the dynamic heterodimerization of HER receptors activates a large repertoire of transforming signaling molecules and pathways that are, to a great

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extent, shared by these HER members. The phosphatidylinositol 3 kinase (PI3-K) and mitogen-activated protein kinase (MAPK) kinase (MEK) are two important signaling molecules diverting the pathways in transducing the mitogenic and survival signals to the nuclei.

Trastuzumab (also known as Herceptin or rhu-mAbHER2) is the humanized form of the murine 4D5 antibody that is directed to the external domain of HER2. Trastuzumab inhibits the growth of a variety of cancer cells overexpressing HER2 in cell culture, animal model, and clinical trials (16–20). Its success in clinical trials with breast cancer has led to US Food and Drug Administration (FDA) approval for its use in the treatment of metastatic breast cancer when combined with chemotherapy (21, 22). Trastuzumab is also active and well tolerated when used as a single agent in the first-line treatment of women with HER2-overexpressing metastatic breast cancer (23, 24). Further investigation is currently under way to define its potential utility in the context of combination treatment of breast cancer with other conventional cytotoxic or biological therapies (25–27).

In contrast to the studies exploring the potential role of the HER2-mediated signals in conferring on breast cancer cells' resistance to chemotherapeutic agents, there are few studies in literature investigating the role of HER2 in the radiosensitivity of cancer cells (28). One recent study using CI-1033, a potent small molecule inhibitor that blocks the kinase activity of all four known members of the EGF receptor family and inhibits the proliferation of HER2-overexpressing breast cancer cell lines, found that there was a synergistic effect when CI-1003 was used with ionizing radiation (29). Another recent study on human head and neck cancer cells reported that trastuzumab sensitized the cells to ionizing radiation (30).

Because of the well-established role of HER2 in breast cancer oncogenesis and the fact that breast cancer currently is the only type of human cancer for which trastuzumab has been approved by the FDA, in the current study, we examined the potential role of HER2 in breast cancer radioresistance, and explored whether trastuzumab may potentiate breast cancer cells to ionizing radiation and what is the major affected downstream pathway responsible for the potential radiosensitization by trastuzumab. We conducted this study using a panel of six breast cancer cell lines expressing different levels of HER2. We found that exposure of the breast cancer cells to trastuzumab markedly sensitized the induction of apoptosis by ionizing radiation in cell lines with high levels of HER2 (BT474, SKBR3, and MDA453), but not in cell lines with low levels of HER2 (MCF7, ZR75B, and MDA468). We further found that MCF7 cells with experimentally elevated levels of HER2 protein showed increased resistance to ionizing radiation-induced apoptosis and increased post-radiation clonogenic survival. Down-regulation of HER2 by trastuzumab in these MCF7HER2 transfectant cells restored cellular radiosensitivity. This HER2-mediated radioresistance in MCF7 cells involves at least two major HER2 downstream pathways, PI3-K/Akt and MEK/MAPK.

Using small molecule inhibitors that specifically inhibit PI3-K or MEK, we found that inhibition of the PI3-K pathway had a stronger effect than inhibition of MEK in sensitizing MCF7HER2 cells to radiation-induced damage, suggesting that inhibition of PI3-K plays a major role in trastuzumab-mediated radiosensitization of breast cancer cells with high levels of HER2.

## Materials and Methods

### Antibodies and Reagents

Antibodies used for detection of HER2 by Western blot analysis were purchased from Oncogene Research Products (Cambridge, MA). Antibodies directed against total Akt, ser473-phosphorylated Akt1, and phosphorylated MAPK p42/p44 were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-MAPK (Erk2) antibody was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti- $\beta$ -actin antibody was from Sigma Chemical Co. (St. Louis, MO). Trastuzumab (Herceptin) was obtained from Genetech, Inc. (San Francisco, CA). LY294002 and PD98059 were obtained from CalBiochem Corp. (San Diego, CA). All other reagents were purchased from Sigma unless otherwise specified.

### Cell Lines and Culture

All breast cancer cell lines (BT474, SKBR3, MDA453, ZR75B, MDA468, MCF7, and MCF7HER2) were grown and routinely maintained in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 units/ml penicillin, and 100  $\mu$ g/ml streptomycin and incubated in a 37°C humidified atmosphere (95% air and 5% CO<sub>2</sub>). The MCF7HER2 cells used in this study were neomycin (G418)-selected pooled MCF7 cells transfected with the full-length HER2 cDNA fragment subcloned in the pcDNA3.0 expression vector (Invitrogen, Carlsbad, CA).

### Ionizing Radiation

Cells grown on Petri dishes were irradiated with  $\gamma$  rays from a high-dose-rate <sup>137</sup>Cs unit (4.5 Gy/min) at room temperature. After irradiation at various doses, the cells were harvested by trypsinization for the various studies described below.

### Clonogenic Assay

Cells were plated in triplicate into 6-cm dishes with densities varying from 300 to 1200 cells/dish (to yield 50–200 colonies per dish) depending on the radiation dose that the cells received. The cells were then cultured in a 37°C, 5% CO<sub>2</sub> incubator for 14 days. Individual colonies (>50 cells/colony) were fixed and stained with a solution containing 0.25% Gentian violet and 10% ethanol for 10 min. The colonies were counted with the FluorChem 8800 Imaging System (Alpha Innotech Corporation, San Leandro, CA) using a visible light source. The plating efficiency (PE) represents the percentage of cells seeded that grow into colonies under a specific culture condition of a given cell line. The survival fraction, expressed as a function of irradiation, was calculated as follows: Survival fraction = colonies counted/(cells seeded  $\times$  PE/100).

### Quantification of Apoptosis by ELISA

An apoptosis ELISA kit (Roche Diagnostics Corp., Indianapolis, IN) was used to quantitatively measure cytoplasmic histone-associated DNA fragments (mononucleosomes and oligonucleosomes) as we previously reported (31). Cell lysate samples with equal amounts of protein (40  $\mu$ g) were used for this photometric enzyme immunoassay, which was performed exactly according to the manufacturer's instructions.

### Western Blot Analysis

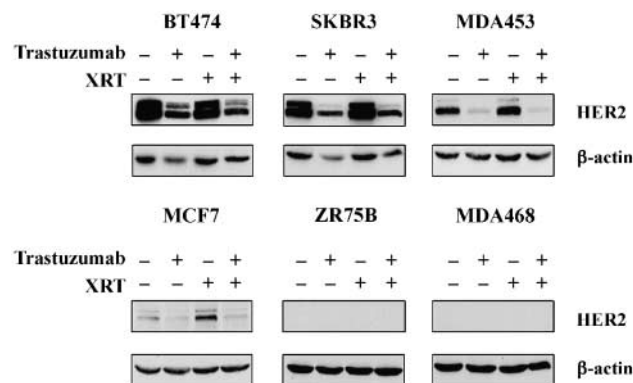
The cells in culture dishes were washed with cold PBS two times and then harvested with a rubber scraper. Cell pellets were lysed with a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% NP40, 50 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , 1 mM phenylmethylsulfonyl fluoride, 25  $\mu$ g/ml leupeptin, and 25  $\mu$ g/ml aprotinin. The lysates were cleared by centrifugation, and the supernatants were collected. Lysate samples with equal amounts of protein (100  $\mu$ g) were assayed for the levels of protein expression by Western blot analysis with the indicated antibodies (32). Specific signals were visualized using the ECL chemoluminescence detection kit (Amersham, Arlington Heights, IL).

## Results

### Down-Regulation of HER2 by Trastuzumab in HER2-Overexpressing Breast Cancer Cells Is Associated with Increased Induction of Apoptosis by Ionizing Radiation

We first used a panel of six breast cancer cell lines with various levels of HER2 to determine whether trastuzumab could sensitize the cells to ionizing radiation-induced apoptosis. Fig. 1 shows the expression levels of HER2 protein and the effect of trastuzumab on the levels of HER2 in these cell lines. Exposure of the cells with intermediate to high levels of HER2 (BT474, SKBR3, and MDA453) to trastuzumab markedly reduced the levels of HER2 in these cells (Fig. 1, top three gels). There were no detectable differences in the levels of HER2 and the efficiency of trastuzumab in down-regulating HER2 between non-irradiated and irradiated cells. In contrast to BT474, SKBR3, and MDA453 cells, MCF7 cells express low levels of HER2. There was a moderate increase in the levels of HER2 in the MCF7 cells following ionizing radiation. Treatment of MCF7 cells with trastuzumab down-regulated HER2 in both non-irradiated and irradiated cells to a similar lower level (Fig. 1, bottom left gel). ZR75B and MDA468 cells have no detectable level of HER2, and there was no change in the level of HER2 with or without ionizing radiation. Exposure of the cells to trastuzumab did not have any effect on the level of HER protein (Fig. 1, bottom center and right gels).

To assess whether trastuzumab may sensitize breast cancer cells to ionizing radiation-induced apoptosis, an apoptosis ELISA was used to quantitatively measure the apoptosis in the panel of cell lines with or without trastuzumab treatment. In contrast to the results with the three cell lines that express low to no detectable levels of

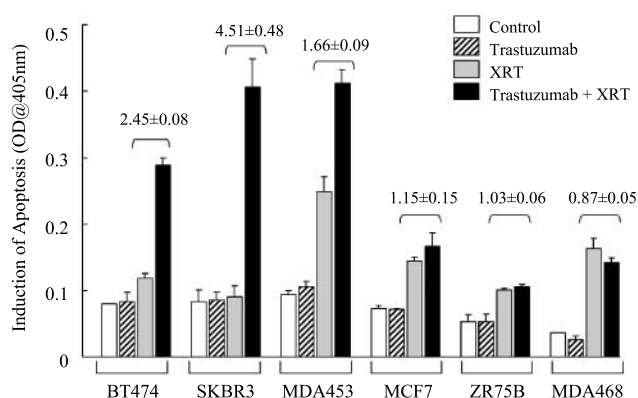


**Figure 1.** Effect of ionizing radiation and trastuzumab on the levels of HER2 in human breast cancer cells. The six indicated breast cancer cell lines with various levels of HER2 were cultured with or without 20 nM trastuzumab for 16 h in 0.5% FBS medium. The cells were then either exposed or not to 10 Gy of radiation, and continuously cultured for an additional 24 h with or without 20 nM trastuzumab as they were before irradiation. After the treatment, the cells were harvested and lysed as described in "Materials and Methods." Lysate samples with equal amounts of protein from each cell line were analyzed for the protein levels of HER2 by Western blot analysis with specific anti-HER2 antibodies. The level of  $\beta$ -actin was used as a protein-loading reference control in each cell line. XRT, radiotherapy.

HER2 (MCF7, ZR75B, and MDA468), exposure of BT474, SKBR3, or MDA453 cells to trastuzumab markedly sensitized these cell lines to ionizing radiation-induced apoptosis (Fig. 2). Compared with the induction of apoptosis by radiation treatment alone (=1 arbitrary unit in each individual cell lines), the values for apoptosis induced by the combination of radiation and trastuzumab treatment were  $2.45 \pm 0.08$  in BT474 cells,  $4.51 \pm 0.48$  in SKBR3 cells, and  $1.66 \pm 0.09$  in MDA453 cells. In contrast, the values were  $1.15 \pm 0.15$  in MCF7 cells,  $1.03 \pm 0.06$  in ZR75B cells, and  $0.87 \pm 0.05$  in MDA468 cells.

### MCF7 Breast Cancer Cells Expressing High Levels of HER2 Protein Showed Increased Radioresistance, Which May Be Resensitized by Trastuzumab

To confirm the results obtained from the correlative studies in the panel of breast cancer cell lines (shown in Figs. 1 and 2), we next conducted experiments in MCF7 cells with and without high levels of transfected HER2 protein (33). Compared with parental or control vector-transfected MCF7 cells, MCF7 cells expressing high levels of HER2 showed higher activity of receptor tyrosine kinase, as shown by increased levels of phosphorylated Akt and phosphorylated MAPK (Fig. 3A). A technical point that needs to be addressed here is that, due to the difference in the affinities of the antibodies used in Western blot analyses for HER2, phosphorylated Akt or phosphorylated MAPK, the magnitude of the increase in the levels of phosphorylated Akt or phosphorylated MAPK may not necessarily be proportional to the magnitude of the increase in HER2 levels. Importantly, there are increases in the levels of phosphorylated Akt and phosphorylated MAPK, indicating that the transfected HER2 was active in



**Figure 2.** Effect of trastuzumab on sensitizing ionizing radiation-induced apoptosis in breast cancer cell lines with various levels of HER2 protein. The six indicated breast cancer cell lines were treated as described in the legend for Fig. 1. Cells were harvested and lysed, and lysate samples with equal amounts of protein of each cell line were subjected to a quantitative ELISA apoptosis assay as described in "Materials and Methods." The numbers in the groups of each cell line indicate the fold increases in XRT-induced apoptosis by trastuzumab (compared with XRT alone). XRT, radiotherapy;  $\pm$ , SD of triplicate samples.

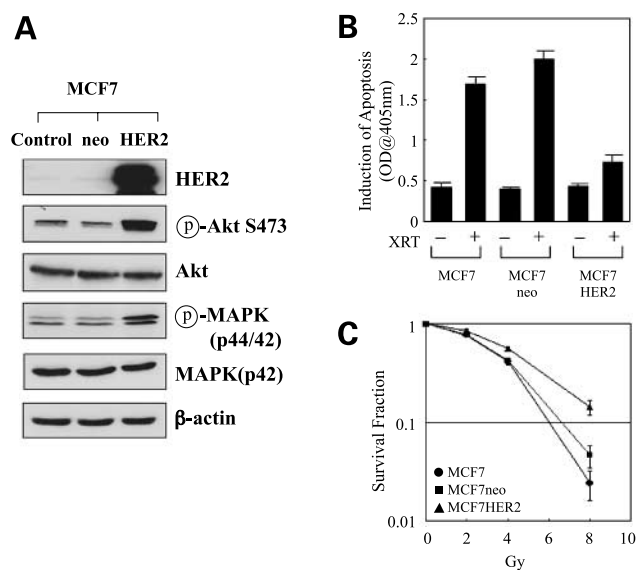
transducing mitogenic signals to its downstream substrates. Compared with parental or control vector-transfected MCF7 cells, MCF7HER2 cells showed markedly increased resistance to ionizing radiation-induced apoptosis (Fig. 3B). This increased resistance was in resonance with the increased clonogenic survival of MCF7HER2 cells following escalating doses of radiation (Fig. 3C). At a radiation dose of 4 Gy, the survival fraction of MCF7HER2 cells was 0.55 compared with 0.42 for both parental and control vector-transfected MCF7 cells. At a radiation dose of 8 Gy, the survival fraction of MCF7HER2 cells was 0.14 compared with 0.02 for parental MCF7 cells and 0.04 for control vector-transfected MCF7 cells.

We next determined whether this increased radioresistance caused by experimental elevation of HER2 in MCF7HER2 cells could be resensitized by treatment of the cells with trastuzumab. Fig. 4 shows the result of the down-regulation of HER2 by trastuzumab in MCF7HER2 cells in both time- and dose-dependent manners. Exposure of the cells to 20 nM trastuzumab for 48 h markedly reduced the level of HER2 in the cells (Fig. 4A). The down-regulation of HER2 in MCF7HER2 was accompanied by lower levels of phosphorylation of Akt and MAPK, the difference being most evident at the 48-h time point (Fig. 4A). Down-regulation of HER2 in MCF7HER2 cells resensitize the cells to radiation-induced apoptosis (Fig. 4B). However, when the cells were measured for clonogenic assays, we found that a 48-h pre-radiation exposure of the cells to trastuzumab was not sufficient to produce noticeable difference between trastuzumab-treated cells and -untreated cells in their clonogenic survival after radiation (data not shown). Thus, we conducted experiments in which trastuzumab was added only shortly before the radiation (1 h) but was rather retained during a 14-day culture period of the clonogenic assays. Compared with the clonogenic survival data of MCF7HER2 cells by ionizing radiation alone, the presence of

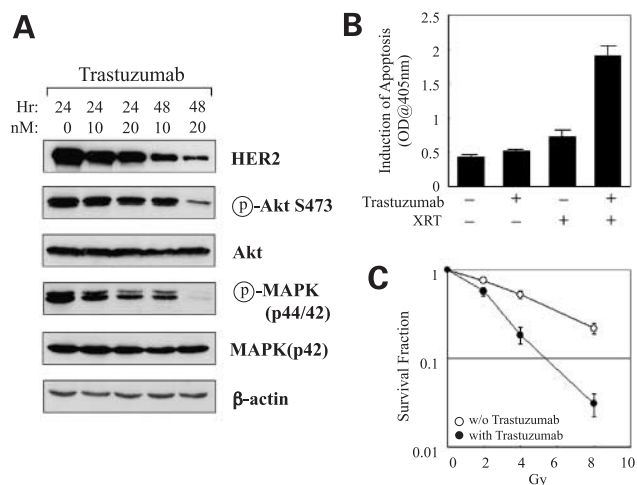
trastuzumab during the post-radiation period markedly reduced the clonogenic survival of the cells; the survival fraction was reduced from 0.22 following 8 Gy of irradiation alone compared with 0.03 when radiation was combined with trastuzumab treatment (Fig. 4C). Of note, the effect of radiation in combination with trastuzumab was normalized to the effect on cells by trastuzumab alone, which reduced the clonogenic survival of MCF7HER2 cells from 100% in untreated cells to  $85 \pm 5\%$  in the 14-day culture period. Our observation indicates that a continued inhibition of HER2-mediated signal transduction is important in suppressing the clonogenesis after radiation.

### Dissection of the Inhibition of PI3-K and MEK Pathways in Trastuzumab-Mediated Radiosensitization in Breast Cancer Cells

The PI3-K/Akt and MEK/MAPK pathways are the two major downstream components in relaying the mitogenic and survival signals into the nuclei following HER2 activation. To evaluate their involvement in trastuzumab-mediated radiosensitization of the breast cancer cells, we conducted experiments to determine whether individual inhibition of PI3-K/Akt or MEK/MAPK alone could produce similar effects as trastuzumab in sensitizing the



**Figure 3.** Role of HER2 in mediating radioresistance in MCF7 cells. In **A**, MCF7 parental cells, MCF7neo cells, and MCF7HER2 grown in regular culture medium were lysed and analyzed by Western blot analysis to determine the protein levels of HER2 and the phosphorylated and total protein levels of Akt and MAPK of the cells. The level of  $\beta$ -actin was used as a protein-loading reference control in each cell line. In **B**, MCF7 parental cells, MCF7neo cells, and MCF7HER2 cells with or without 10 Gy of radiation were cultured in 0.5% charcoal-stripped bovine serum for 24 h. The cells were lysed, and lysate samples with equal amounts of protein were subjected to a quantitative ELISA apoptosis assay as described in "Materials and Methods." In **C**, following various doses of radiation (0, 2, 4, and 8 Gy), MCF7 parental cells, MCF7neo cells, and MCF7HER2 were individually seeded and cultured for 14 days in 10% FBS medium. After normalization with the numbers of the cells seeded in individual groups, the percentages of surviving colonies of MCF7 parental cells, MCF7neo cells, and MCF7HER2 cells were plotted against the doses of ionizing radiation received by the cells. XRT, radiotherapy.  $\pm$ , SD of triplicate samples.

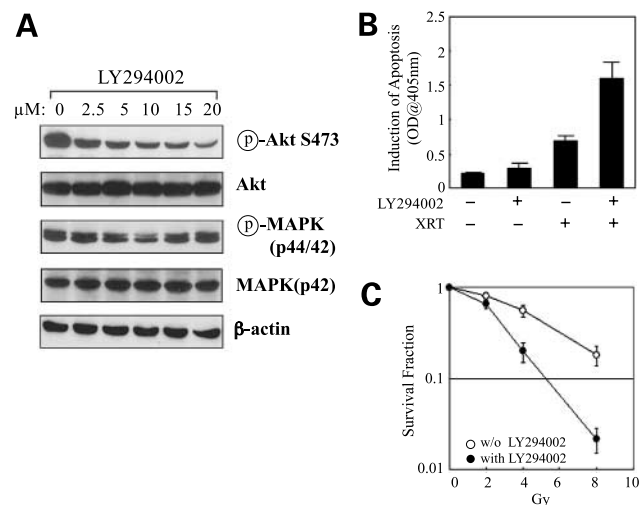


**Figure 4.** Effect of trastuzumab on radiation-induced apoptosis and on post-radiation clonogenic survival in MCF7HER2 cells. In **A**, MCF7HER2 cells were untreated or treated with 10 or 20 nM trastuzumab in medium containing 0.5% charcoal-stripped serum for 24 or 48 h as indicated. The cells were then lysed and examined for the protein levels of HER2 as well as the phosphorylated and total protein levels of Akt and MAPK by Western blot analysis with specific antibodies. The level of  $\beta$ -actin was used as a protein-loading reference control in each cell line. In **B**, at 16 h into a 40-h incubation period, MCF7HER2 cells in medium with 0.5% charcoal-stripped bovine serum with or without 20 nM trastuzumab were either irradiated or not with 10 Gy of radiation and then continuously cultured for the remaining 24 h before harvest. Cell lysates were prepared, and lysate samples with equal amounts of protein were analyzed by the ELISA apoptosis assay as described in "Materials and Methods." In **C**, before exposure to various doses of radiation (0, 2, 4, and 8 Gy), MCF7HER2 cells were exposed to 20 nM trastuzumab for a short period (1 h). The irradiated cells were then seeded into cultures, allowed to attach overnight, and cultured for 14 days in culture medium containing 10% FBS with or without 20 nM trastuzumab. The radiosensitization effect of trastuzumab was normalized by the effect of trastuzumab alone on the clonogenic survival, which reduced from 100% in untreated cells to  $85 \pm 5\%$ . Clonogenic survival fractions were calculated by dividing the number of colonies counted by the corresponding number of cells seeded as described in "Materials and Methods." XRT, radiotherapy.  $\pm$ , SD of triplicate samples.

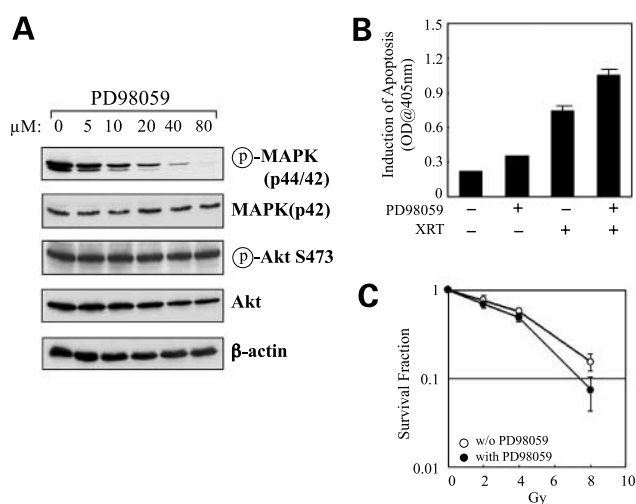
MCF7HER2 cells to ionizing radiation. The compound LY294002 specifically inhibits the activity of PI3-K, whereas PD98059 specifically inhibits MEK. We first confirmed their relative specificities in the MCF7HER2 cells. Exposure of MCF7HER2 cells to LY294002 led to an LY294002 dose-dependent reduction in the level of phosphorylated Akt, a well-known downstream substrate of PI3-K, without affecting the level of phosphorylated MAPK, indicating the specificity of this compound in inhibiting the activity of PI3-K/Akt in MCF7HER2 cells (Fig. 5A). Compared with the results following ionizing radiation alone or LY294002 treatment alone, pre-exposure of MCF7HER2 cells to LY294002 sensitized the cells to radiation-induced apoptosis (Fig. 5B). When the cells were assayed for clonogenic survivals, similar to the scenario with trastuzumab treatment, this treatment regimen was not sufficient to produce remarkable difference between the two groups (radiation alone and radiation plus 48 h pretreatment with LY294002) (data not shown). We thus conducted a similar clonogenic assay as in Fig. 4C, allowing LY294002 to be added only shortly before the radiation (1 h) but were present during a

14-day culture period of the clonogenic assays. We found that, when 8 Gy of radiation was combined with LY294002 treatment, the survival fraction of MCF7HER2 cells was 0.02, compared with a survival fraction of 0.18 following 8 Gy of radiation without the presence of LY294002 (Fig. 5C). Again of note, the effect of radiation and LY292002 combination treatment was normalized to the effect caused by treatment of the cells with LY294002 alone, which reduced the clonogenic survival from 100% in untreated cells to  $73 \pm 11\%$ .

Compared to the results of inhibition of PI3-K/Akt on radiosensitization shown in Fig. 5, experiments on the effects of PD98059 showed a lack of conviction of this pathway in modulating radiosensitivity in MCF7HER2 cells (Fig. 6). As expected, exposure of MCF7HER2 cells to PD98059 showed a marked reduction in the level of phosphorylated MAPK without affecting the level of phosphorylated Akt (Fig. 6A), indicating a relatively specific activity of this compound on MEK. However, compared with the effect of LY294002 on sensitizing radiation-induced apoptosis shown in Fig. 5B, similar treatment of the MCF7HER2 cells to PD98059 was less



**Figure 5.** Effect of PI3-K inhibition on radiation-induced apoptosis and on post-radiation clonogenic survival in MCF7HER2 cells. In **A**, MCF7HER2 cells were treated with indicated doses of LY294002 alone in medium containing 0.5% charcoal-stripped bovine serum overnight (16 h). The cells were then lysed and examined for the phosphorylated and total protein levels of Akt and MAPK by Western blot analysis with specific antibodies. The level of  $\beta$ -actin was used as a protein-loading reference control in each cell line. In **B**, at 16 h into a 40-h incubation period, MCF7HER2 cells in medium with 0.5% charcoal-stripped bovine serum with or without 5  $\mu$ M LY294002 were either irradiated or not with 10 Gy of radiation and then continuously cultured for the remaining 24 h before harvest. Cell lysates were prepared, and lysate samples with equal amounts of protein were analyzed by the ELISA apoptosis assay as described in "Materials and Methods." In **C**, before exposure to various doses of radiation (0, 2, 4, and 8 Gy), MCF7HER2 cells were exposed to 5  $\mu$ M LY294002 for 1 h. The irradiated cells were then seeded into cultures, allowed to attach overnight, and cultured for 14 days in culture medium containing 10% FBS with or without 5  $\mu$ M LY294002. The radiosensitization effect of LY294002 was normalized by the effect of LY294002 alone on the clonogenic survival, which reduced from 100% in untreated cells to  $73 \pm 11\%$ . Clonogenic survival fractions were calculated by dividing the number of colonies counted by the corresponding number of cells seeded as described in "Materials and Methods." XRT, radiotherapy.  $\pm$ , SD of triplicate samples.



**Figure 6.** Effect of MEK inhibition on radiation-induced apoptosis and on post-radiation clonogenic survival in MCF7HER2 cells. In **A**, MCF7HER2 cells were treated with the indicated doses of PD98059 alone in medium containing 0.5% charcoal-stripped bovine serum overnight (16 h). The cells were then lysed and examined for the phosphorylated and total protein levels of Akt and MAPK by Western blot analysis with specific antibodies. The level of  $\beta$ -actin was used as a protein-loading reference control in each cell line. In **B**, at 16 h into a 40-h incubation period, MCF7HER2 cells in medium with 0.5% charcoal-stripped bovine serum with or without 10  $\mu$ M PD98059 were either irradiated or not with 10 Gy of radiation and then continuously cultured for the remaining 24 h before harvest. Cell lysates were prepared, and samples with equal amounts of protein were analyzed by the ELISA apoptosis assay as described in "Materials and Methods." In **C**, before exposure to various doses of radiation (0, 2, 4, and 8 Gy), MCF7HER2 cells were exposed to 10  $\mu$ M PD98059 for 1 h. The irradiated cells were then seeded into cultures, allowed to attach overnight, and cultured for 14 days in culture medium containing 10% FBS with or without 10  $\mu$ M PD98059. The radiosensitization effect of PD98059 was normalized by the effect of PD98059 alone on the clonogenic survival, which reduced from 100% in untreated cells to  $76 \pm 5\%$ . XRT, radiotherapy.  $\pm$ , SD of triplicate samples.

potent in sensitizing the cells to radiation-induced apoptosis (Fig. 6B). Furthermore, clonogenic survival of MCF7HER2 cells that was conducted in a way similar to the experiments shown in Figs. 4C and 5C, indicated that inhibition of MEK with PD98059 had only minimal effect on the clonogenic survival of the irradiated cells (Fig. 6C). After the data were normalized to the effect on clonogenic survival by PD98059 alone, which reduced the clonogenic survival of the cells from 100% in untreated cells to  $76 \pm 5\%$ , the two curves were basically overlapping, with modest difference observed only at 8 Gy. These results suggest that, although inhibition of MEK with PD98059 reduced clonogenic survival of MCF7HER2 cells, it has no recognized effect on sensitizing cells to radiation. Taken together, these observations indicate that the radiosensitization by trastuzumab was mainly mediated through inhibition of the PI3-K pathway.

## Discussion

Sensitization of human cancer cells to radiation by targeting the EGF receptor family tyrosine kinases is being recognized

as a promising novel approach for enhancing the therapeutic effect of radiation (34). Studies testing this hypothesis in literature so far, however, have been limited primarily to the inhibition of EGF receptor, the founding member of this family. We and others reported that treatment of human cancer cells that overexpress the EGF receptor with either EGF receptor-blocking monoclonal antibodies (such as cetuximab, also known as Erbitux or C225) or small molecule EGF receptor inhibitors (such as gefitinib, also known as Iressa or ZD1839) markedly sensitized the cells to the cytotoxic effect of ionizing radiation both *in vitro* and *in vivo* (35–41). Additional experimental approach using a dominant negative form of the EGF receptor (CD533) showed similar results in preclinical studies (42). These important preclinical studies with encouraging results have prompted the current clinical trials for potential enhancement of the therapeutic effects of radiation combined with Erbitux or Iressa treatment in head and neck cancer patients, 90% of whom have overexpression of the EGF receptor (43). In contrast, there are only limited studies exploring whether inhibition of HER2 could similarly sensitize HER2-overexpressing cancer cells to radiation therapy. Because breast cancer is one of the major types of human cancer overexpressing HER2, and because trastuzumab is already approved by FDA in a regimen of combination treatment with chemotherapy for metastatic breast cancer, in the current study, we investigated whether trastuzumab could sensitize human breast cancer to the apoptosis induced by ionizing radiation and the major downstream signal pathway(s) involved in this process.

We found a HER2 level-dependent sensitization to radiation-induced apoptosis by trastuzumab in a panel of breast cancer cell lines. This radiosensitization by trastuzumab was further demonstrated by reduced clonogenic survival of the breast cancer cells after radiation. Because of the extensive genetic heterogeneity and the complexity of the biochemical signal transduction pathways among different cancer cell lines, there is no single molecule that may act alone in determining cellular resistance to radiotherapy. Results from the correlative studies shown in the Figs. 1 and 2 may thus not necessarily support a conclusion that overexpression of HER2 in breast cancer cells leads to radioresistance. We therefore extended our study for a causal relationship by comparing the radiosensitivity in MCF7 cells transfected with either control vector or HER2 expression vector. Experimental elevation of HER2 levels in the MCF7 breast cancer cell line (which normally expresses low levels of HER2) caused increased resistance of the cells to ionizing radiation-induced apoptosis and increased clonogenic survival after radiation. Furthermore, we demonstrated that down-regulation of HER2 by trastuzumab in these MCF7HER2 cells restored the radiosensitivity of the cells. Our data thus strongly indicate that HER2 is an ideal target for sensitizing breast cancer cells to ionizing radiation. The mechanism of such an effect by trastuzumab was attributed primarily to the inhibition of the PI3-K/Akt downstream signal pathway, rather than to that of another important downstream signal pathway, the MEK/MAPK-mediated signal transduction.

Earlier studies have reported that irradiation of cells may activate the HER tyrosine kinase family and trigger subsequent signal transduction (42, 44, 45). This radiation-induced activation of the HER family tyrosine kinases is receptor expression pattern dependent rather than ligand dependent, compared with the hierarchic activation of HER members by their respective specific ligands (45). From a mechanistic point of view, the radiation-induced activation of the HER tyrosine kinase family is likely an adaptive response (as a self-protective mechanism) to radiation-induced damage or stress. We did not find activation of the HER2 pathway in the experimental conditions in our studies (data not shown); however, we did find, in our repeated experiments, that the levels of HER2 were slightly up-regulated in MCF7 cells that were irradiated compared with non-irradiated cells (Fig. 1). This up-regulation of HER2 was not noted in other cell lines in our study, and this could be explained as a failure to discern by Western blot analysis slight increase in HER2 levels after irradiation in cell lines already overexpressing high levels of HER2 (the BT474, SKBR2, and MDA453 cells). However, up-regulation of HER2 after irradiation was not observed either in cell lines that do not express detectable levels of HER2 (such as MDA468 and ZR75B cells). Thus, the slight increase in the HER2 level after irradiation could be cell type specific, limited only to MCF7 cells or to cell lines expressing at least a detectable level of HER2 or HER3.

Several recent studies exploring the downstream mediators of the HER tyrosine kinase family have also shown a potential role for the PI3-K/Akt pathway in mediating radioresistance in cancer cells (46–48). Our current study is in resonance with the findings in our recent publication that overexpression of a constitutively active Akt in MCF7 cells conferred increased resistance to radiation on these cells (49). In our current study, we found that treatment of MCF7HER2 cells with trastuzumab reduced activities of PI3-K and MEK/MAPK, and selective inhibition of the PI3-K/Akt pathway with LY294002 during the post-radiation period appeared to be more effective than selective inhibition of MEK/MAPK with PD98059 in the MCF7HER2 cells. Taken together, our data indicate that inhibition of the PI3-K/Akt pathway may be the major mechanism in reducing the clonogenic survival of the cells following radiation.

A caveat to the approaches we used in current studies is that, although both LY294002 and PD98059 showed relative specificity in inhibiting PI3-K and MEK, respectively, there were still possibilities that the two compounds may inhibit additional kinases other than PI3-K or MEK. Although radiosensitization by inhibiting the MEK/MAPK was minimal, it might be still interesting to determine via a genetic/transfection approach whether expression of constitutively active MEK would significantly alter the sensitivity of MCF7 cells to radiation.

In summary, our results indicate that HER2 is an important determinant conferring resistance on breast cancer cells to radiation. Trastuzumab is currently approved by the FDA only for treatment of patients with chemotherapy-resistant metastatic breast cancer. The

results of our current study support clinical application of trastuzumab for radiosensitization of breast cancer patients. Our observation that PI3-K/Akt plays an important role in HER2-mediated radioresistance also supports novel molecule-targeted approaches for sensitizing human breast cancer cells to radiotherapy with novel inhibitors that specifically inhibit PI3-K/Akt.

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