Monoclonal Antibody to HER-2/neuReceptor Modulates Repair of Radiation-induced DNA Damage and Enhances Radiosensitivity of Human Breast Cancer Cells Overexpressing This Oncogene

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ABSTRACT

The management of human breast cancer frequently includes radiation therapy as an important intervention, and improvement in the clinical efficacy of radiation is desirable. Overexpression of the HER-2 growth factor receptor occurs in 25–30% of human breast cancers and correlates with poor clinical outcome, including earlier local relapse following conservative surgery accompanied by radiation therapy. In breast cancer cells with overexpression of HER-2 receptor, recombiant humanized monoclonal antibodies (rhuMabs) to HER-2 receptors (rhuMab HER-2) decrease cell proliferation in vitro and reduce tumor formation in nude mice. Therapy with rhuMab HER-2 enhances tumor sensitivity to radiation at doses of 1–5 Gy, exceeding remission rates obtained with radiation alone. This benefit is specific to cells with HER-2 overexpression and does not occur in cells without overexpression. Treatment of cells with radiation (2–4 Gy) alone provokes a marked increase in unscheduled DNA synthesis, a measure of DNA repair, but HER-2-overexpressing cells treated with a combination of rhuMab HER-2 and radiation demonstrate a decrease of unscheduled DNA synthesis to 25–44% of controls. Using an alternate test of DNA repair, i.e., radiation-damaged or undamaged reporter DNA, we introduced a cytomegalovirus-driven β-galactosidase into HER-2-overexpressing breast cancer cells that had been treated with rhuMab HER-2 or control. At 24 h posttransfection, the extent of repair assayed by measuring reporter DNA expression was high after high exposure to radiation alone but significantly lower in cells treated with combined radiation and rhuMab HER-2 therapy. To further characterize effects of rhuMab HER-2 and the combination of antibody and radiation on cell growth, analyses of cell cycle distribution were performed. Antibody reduces the fraction of HER-2-overexpressing breast cancer cells in S phase at 24 and 48 h. Radiation treatment is also known to promote cell cycle arrest, predominantly at G1, with low S-phase fraction at 24 and 48 h. In the presence of rhuMab HER-2, radiation elicits a similar reduction in S phase at 24 h, but a significant reversal of this arrest appears to begin 48 h postradiation exposure. The level of S-phase fraction at 48 h is significantly greater than that found at 24 h with the combined antibody-radiation therapy, suggesting that early escape from cell cycle arrest in the presence of an antibody may not allow sufficient time for completion of DNA repair in HER-2-overexpressing cells. Because it is well known that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest, we also assessed the activity of this critical mediator of the cellular response to DNA damage. The results show induction of p21WAF1 transcripts and protein product at 6, 12, and 24 h after radiation treatment; however, increased levels of p21WAF1 transcript and protein are not sustained in HER-2-overexpressing cells exposed to radiation in the presence of rhuMab HER-2. Although transcript and protein levels increase at 6–12 h, they are both diminished by 24 h. Levels of p21WAF1 transcript and protein at 24 h are significantly lower than in cells treated by radiation without antibody. A reduction in the basal level of p21WAF1 transcript also occurred after 12–24 h exposure to antibody alone. The effect of HER-2 antibody may be related to tyrosine phosphorylation of p21WAF1 protein.

Tyrosine phosphorylation of p21WAF1 is increased after treatment with radiation alone, but phosphorylation is blocked by combined treatment with antireceptor antibody and radiation. This dysregulation of p21WAF1 in HER-2-overexpressing breast cells after treatment with rhuMab HER-2 and radiation appears to be independent of p53 expression levels but does correlate with reduced levels of mdm2 protein. These data indicate that human breast cancer cells damaged by radiation may be especially vulnerable to injury if they are also deprived of essential signal transduction pathways provided by the HER-2 growth factor receptor pathway.

INTRODUCTION

In patients with breast cancer, adjuvant radiation therapy is an important therapeutic intervention following breast conservation surgery (1, 2). Radiation therapy is also recommended after total mastectomy and lymph node dissection for women with large primary cancers or extensive lymph node metastasis. Despite these interventions, however, local recurrence still accounts for an estimated 30–50% of all first recurrences in subgroups of patients (2). Given these data, it is clear that improvement in the efficacy of adjuvant radiation therapy is desirable. The response of breast malignancies to ionizing radiation is commonly the result of DNA injury. Human cells exhibit complex responses to DNA damage, including activation of genes involved in cell cycle arrest, DNA repair, and apoptosis. Recent findings suggest that the cellular response to DNA damage is markedly impaired by deprivation of essential growth factors or by blockade of growth factor receptors (2, 3). Signal transduction pathways mediated by receptor tyrosine kinases and protein kinase C appear to be important for the induction of many of the genes related to key cellular functions that permit the cell to survive a dose of radiation. Specific blockade of these pathways in tumor cells may provide attractive targets for increasing the cytotoxic effects of radiation. Growth factors and their receptors also play a pivotal role in the regulation of human breast cell growth and differentiation. Among growth factor receptors, the most frequently implicated in the pathogenesis of human breast cancer have been members of the erb B receptor family, especially the HER-2 (erb B2) protein, a M, 185,000 transmembrane receptor tyrosine kinase encoded by HER-2/neu proto-oncogene (4). HER-2 is amplified and overexpressed in 25–30% of human breast cancers (5–8), and overexpression of the structurally unaltered HER-2 gene leads to neoplastic transformation of NIH-3T3 cells (9–11) and immortalized human breast cells (8, 12), indicating that this alteration plays a pathogenic role in promoting tumorigenicity of nonmalignant cells. Monoclonal antibodies against the extracellular domain of HER-2 specifically inhibit the growth of human breast carcinoma cells overexpressing the HER-2 gene product (8). Amplification of the HER-2 oncogene has also been shown to correlate with distant relapse and overall patient survival in patients with breast cancer (5–8, 13–15). More recently, overexpression of
HER-2 has been found to correlate with the risk for local relapse in patients treated only with conservative surgery and radiation (16).

A potential correlation between HER-2 receptor overexpression and sensitivity to DNA damage from chemotherapeutic drugs and radiation, derived from several laboratory studies, may prove to be clinically useful. The initial studies implicating an interaction between inhibitors of type I receptor tyrosine kinases by antibodies and response to DNA-damaging agents were part of the studies of the EGF receptor. These studies indicated a synergistic effect between antibodies to EGF receptor and the chemotherapeutic drug, cisplatin (17). This combined treatment elicited a significant reduction in both the number and size of epidermoid cancers grown as s.c. xenografts in athymic mice (see also Ref. 18). EGF is also reported to enhance the sensitivity of human squamous carcinoma cells to radiation therapy (19). The potential role of proto-oncogenes for EGF and HER-2 receptors in the modulation of sensitivity to radiation has also been suggested from limited laboratory studies (20, 21). We have shown that blockade of the HER-2 receptor in human breast cancer cells using an anti-HER-2 antibody promotes a synergistic antitumor effect when combined with the DNA-damaging drug, cisplatin (22), and that the signal generated by antibody binding to HER-2 receptors may block DNA repair in HER-2-overexpressing cells (23). Nerve growth factor is also known to reduce DNA repair induced by UV radiation and to slow removal of DNA adducts induced by benzo(a)pyrene in neuroblastoma cells (24). However, the specific molecular pathways used by cells for suppression of DNA repair, which are triggered by ligand (or antireceptor antibody) interactions, remain unclear. The tumor suppressor gene product p53 is a critical mediator of the cellular response to DNA damage. Both cell cycle arrest (25) and programmed cell death (26) after DNA damage due to ionizing radiation are closely linked to p53 function. Recent data further suggest that p53 may be critical in the repair of DNA damage (27).

Many of the effects of p53 can be attributed to the function of downstream p53-regulated genes, including p21WAF1, also known as Cip1, sd1, or CAP20, that codes for a M, 21,000 protein (p21WAF1; Ref. 28). Cell cycle checkpoints on progression of cells through the G1 and into S phase are controlled by protein kinases, the CDKs, as reviewed by Sherr (29). These CDKs are regulated, in turn, by growth factor signaling pathways (3, 30). Recent reports show p53-independent activation of p21WAF1 by MAP kinase signaling (31). In addition, withdrawal of growth factors in vitro has been associated with down-regulation of p21WAF1 expression and enhanced cell killing in response to DNA damage (32).

Here, we tested the hypothesis that DNA damage induced by external-beam radiation can be combined with agents designed to act selectively against cells in which the HER-2 receptor pathway is altered for therapeutic advantage. Cross-communication between DNA damage response pathways involving p21WAF1 and growth factor signaling pathways, i.e., HER-2, is evaluated in breast cancer cells with HER-2 overexpression.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The human breast carcinoma cell line, SKBR3, constitutively overexpresses the HER-2 gene and was obtained from the American Type Culture Collection (Manassas, VA). Stable retroviral transfectants of MCF-7 human breast cancer cells that overexpress the HER-2 receptor (MCF-7/HER-2) have been prepared (11, 22). Cells infected with a control retroviral vector not containing the HER-2 gene (MCF-7/CON) as well as parental cells not infected with retrovirus (MCF-7/PAR) were used as controls in in vitro and in vivo experiments. All cells were routinely cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 2 mM freshly added glutamine, and 1% penicillin G-streptomycin-fungizone solution (Irvine Scientific, Santa Ana, CA).

Anti-HER-2 Receptor Monoclonal Antibodies. rhuMAB to HER-2 receptor (rhuMAB HER-2; generously provided by Genentech, South San Francisco, CA), derived from the murine monoclonal antibody 4D5, is directed to an extracellular epitope of HER-2 receptor. Antibodies were prepared as described in detail elsewhere (32, 33). Control experiments were conducted with nonspecific IgG of the same class and isotype to verify the specificity of any observed effects.

Tumor Formation in Nude Mice. Human breast cancer cells were inoculated s.c. at 5 × 10^5 cells/animal in the hind thighs of 3-month-old female athymic mice. Prior to inoculation, mice were primed for 10–14 days with 17β-estradiol applied s.c. in a biodegradable carrier-binder (1.7 mg of estradiol per pellet) to promote growth of these estrogen-dependent breast cancer cells. Tumor nodules were monitored by dimension measurements (in mm). Five to six animals were included in each treatment group, with randomization by weight and tumor nodule size at the start of each experiment. Antibody treatment was initiated when tumors grew to >50 mm^3 in size in one set of experiments or to >350 mm^3 in size in independent studies. Monoclonal antibody and control solutions were administered by i.p. injection. The rhuMAB HER-2 antibody was given at a dose of 5 or 10 mg/kg animal weight in three doses at 4-day intervals (over 12 days). Control injections included human IgG1 (5 or 10 mg/kg), given on a similar treatment protocol. Radiation treatments were designed to mimic protocols in use in the clinic, and radiation to tumors growing s.c. on a peripheral rear extremity was performed with special planning to avoid inappropriate radiation exposure to non-tumor-bearing sites. Mice were sacrificed for pathological examination under supervision of the institutional veterinarian. Euthanasia technique was cervical dislocation, which is consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association. Tumors were analyzed to confirm HER-2 expression by immunohistochemistry as described elsewhere (6).

Measurement of Cell Cycle Phase Distribution. MCF-7 cells with HER-2 overexpression were plated on plastic at low density and allowed to adhere. After 48 h, the monolayers were washed with PBS and incubated in medium with 1% DCC-stripped FCS to arrest cell growth. After 48 h, this medium was replaced with medium containing either 1% DCC-treated FCS or 10% FCS with or without 200 μg/ml monoclonal antibody to the HER-2 receptor. Cells in medium with 10% FCS but without antibody were treated with 4 Gy of radiation, and cells with 10% FCS plus 200 μg/ml antibody were similarly treated. Cells from all treatment groups were incubated further for 24 or 48 h. Cells were then prepared for cell cycle analysis by established methods, using DNA staining with propidium iodide and flow cytometry (34). The proportion of cells in S phase was quantitated to assess effects of DNA damage and modulation by the antibody (34).

Measurement of DNA Repair. UDS, a type of DNA repair that is non-semiconservative in nature, is a well-established measure of the effects of ionizing radiation (22, 35). Using methods detailed previously (22), we measured UDS by autoradiographic approaches (36) in parent and daughter cells with and without exposure to antireceptor antibody and radiation, either given alone or in combination. Treatment with antibody (200 μg/ml) occurred for 4 h before exposure to radiation. An alternate measure of DNA repair involved introduction of radiation-damaged or reporter DNA into breast tumor cells. Prior to transfection, a reporter DNA, CMV-driven β-galactosidase (pCMV-β; Clontech), was prepared with or without exposure to ionizing radiation in vitro, using methods reported previously (37). For transfection experiments, cells were plated 24 h prior to transfection, and transfections with internal controls to measure transfection efficiency were carried out as described previously (37, 38). Undamaged or radiation-damaged DNA (1.5 μg) was used in these transfection experiments. At 24 h posttransfection, the extent of repair was assayed by measuring reporter DNA expression. The transfected cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase, to distinguish between β-galactosidase-positive and -negative cells. In

1 The abbreviations used are: EGF, epidermal growth factor; CDK, cyclin-dependent kinase; MAP kinase, mitogen-activated protein kinase; rhuMABs, recombinant humanized monoclonal antibody; DCC, dextran-coated charcoal; UDS, unscheduled DNA synthesis; CMV, cytomegalovirus; PCNA, proliferating cell nuclear antigen.
the presence of substrate, cells expressing bacterial β-galactosidase appeared blue, and the percentage of stained cells was quantitated.

Measurement of p21WAF1 Transcript and Protein Levels. Transcripts of p21WAF1 were determined by Northern blot analysis, using established protocols (5, 6, 28, 38). In brief, breast cancer cells with and without HER-2 overexpression were treated with or without rhuMAb HER-2 for 4–24 h before exposure to radiation (0–20 Gy). Cells were then maintained for 6, 12, or 24 h prior to harvesting and processing for collection of RNA. After Northern blot analysis, the resulting blots were hybridized with p21WAF1 cDNA (2.1 kb, Ref. 28). In control studies, some blots were hybridized with cDNA for human p53 (2.0-kb BamH1 fragment) or human cyclin D (American Type Culture Collection). These probes were treated with a random priming method (39).

Western analyses of the level of p21WAF1 protein in breast cancer cells were conducted with methods described previously (38). We assessed p21WAF1 protein response to DNA damage in breast cancer cells in the presence and absence of growth factor receptor antibody. Breast cancer cells with and without HER-2 overexpression were treated with 100 μg/ml rhuMAb HER-2 for 4–24 h before exposure radiation. Cells were then maintained for 6 and 24 h prior to harvesting and processing of cell lysates for electrophoresis (38). Immunoblotting was performed with MAb 6B6 with specificity for human p21WAF1 (PharMingen). In other control studies, immunoblotting was also performed with monoclonal antibodies to PCNA (Santa Cruz Biotechnology), p53 (PAhB1801, AB-2; Oncogene Science), mdm2 (Oncogene Research Products), and bcl-2 (Transduction Laboratories).

RESULTS

Sensitivity of Breast Cancer Cells with HER-2 Overexpression to Radiation and Effects of Anti-HER-2 Antibody. We have directly compared the in vitro radiation sensitivities of parental breast cancer cells with normal expression levels of HER-2 to that of matched daughter cells containing HER-2 overexpression (Fig. 1A). Survival curves were obtained after treatment of cells with graded doses of ionizing radiation at a dose rate of 1 Gy/min with doses of 0, 1, 2, 4, and 5 Gy. After the radiation treatment, cells were placed into 35-mm dishes and cultured for 14 days, with the surviving fractions quantitated at day 14 (20). Irradiation of MCF-7/HER-2 cells in vitro resulted in a 0.10 (the dose required to reduce cell survival to 10%) that was increased by ~25% relative to MCF-7/control cells (Fig. 1A). These analyses suggest a potential biological role of the HER-2 oncogene in resistance to radiation treatment. Alternatively, this apparent difference in sensitivity could be the result of a differential growth rate because HER-2-overexpressing cells have been found to exhibit more rapid growth than control cells following the initial response to chemotherapy (40).

To evaluate the in vitro effect of radiation combined with antibodies to HER-2, we conducted studies using the transfected MCF-7 cells (Fig. 1A) as well as the naturally HER-2-overexpressing SKBR3 breast cancer cells (Fig. 1B). Cells cultivated either on plastic (MCF-7/HER-2 cells) or in soft agar plates (SKBR3 cells) with or without rhuMAb HER-2 were treated with or without radiation prior to incubation in vitro (see Fig. 1). These data show that anti-HER-2 receptor antibody enhances radiation-induced killing of human breast cancer cells with HER-2 overexpression.

In Vivo Effects of Ionizing Radiation Combined with Anti-HER-2 Antibody on Human Breast Cancer Xenografts. To evaluate the efficacy of radiation therapy with rhuMAb HER-2 on the growth of MCF-7/HER-2 xenografts, we inoculated cells into estrogen-primed female athymic mice for 14 days and allowed them to grow to 50–100 mm3 prior to randomization into four groups. Treatment groups included control human IgG1 at 30 mg/kg (Fig. 2A, CON), radiation at 4 Gy with human IgG1 (RT), rhuMAb HER-2 at 30 mg/kg (MAb), or combined radiation/rhuMAb (RT/MAb) therapy (Fig. 2A). Doses of antibody or IgG1 were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received a treatment 4 h after administration of antibody or control IgG1 on days 1, 4, and 7. Tumor nodules were monitored through day 49. The effect of repeated doses of rhuMAb HER-2 with or without ionizing radiation on tumor volume in the various groups was measured (Fig. 2A). In mice receiving low doses of radiation with control IgG (Fig. 2A, RT), mean tumor volumes were not significantly reduced over the 7-week observation period, and no tumor remissions were observed compared to controls (CON). Tumors treated with rhuMAb HER-2 alone (MAb) also failed to show significant growth reduction compared to controls and, again, no tumor remissions were noted. In contrast, combined radiation-antibody therapy produced marked reduction in tumor volumes over the 7-week treatment period compared to control or either treatment alone (P < 0.001). Moreover, all animals that received both rhuMAb HER-2 and radiation (Fig. 2A, RT/MAb) had complete tumor remissions.

To better evaluate the in vivo efficacy of radiation therapy with rhuMAb HER-2 on the growth of larger MCF-7/HER-2 xenografts, cells were inoculated in estrogen-primed female athymic mice and allowed to form 350–400-mm3 tumors prior to randomization into four groups. Treatment groups included control human IgG1 at 15 mg/kg (Fig. 2B, CON), radiation at 8 Gy with human IgG1 (RT), rhuMAb HER-2 at 15 mg/kg (MAb), and combined radiation/rhuMAb (RT/MAb) therapy. Doses of antibody or IgG1 were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received a treatment 4 h after antibody or control IgG1 only on day 1. Tumor nodules were monitored through day 15. A lower dose of rhuMAb HER-2 with or without ionizing radiation was used, and
MCF-7/HER-2 cells were injected s.c. at 5 × 10^7 cells per nude mouse. After 14 days, mice were randomized on day 0 to groups of six animals on the basis of body weight and tumor nodule size. Treatment groups included human IgG1 control at 30 mg/kg (CON, ■), radiation at 4 Gy with human IgG1 (RT, △), rhuMAb HER-2 at 30 mg/kg (MAb, ●), or combined radiation/rhuMAb (RT/MAb, ◆) therapy. Doses of antibody or IgG1 indicated above were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received a 4-Gy treatment at 4 h after administration of antibody or IgG1 on days 1, 4, and 7 only. Tumor nodules were monitored to day 49. On postmortem examination, no residual tumor cells were found by light microscopy in the RT/MAb treatment group, but cancer cells were found in the s.c. nodules of mice from all other treatment groups. B, MCF-7/HER-2 cells were injected s.c. at 5 × 10^7 cells per nude mouse. After 35 days, mice were randomized on day 0 to groups of three to five animals on the basis of body weight and tumor nodule size, with tumors ranging in size from 350 to 400 mm^3. Treatment groups included human IgG1 control at 15 mg/kg (CON), radiation at 8 Gy with human IgG1 (RT), rhuMAb HER-2 at 15 mg/kg (MAb), or combined radiation/rhuMAb (RT/MAb) therapy. Doses of antibody or IgG1 indicated above were administered in divided doses on days 1, 4, and 7. Those groups treated with radiation received an 8-Gy treatment at 4 h after administration of antibody or IgG1 on day 1 only. Tumor volumes were recorded at day 1 and reassessed at 15 days.

Effects on tumor volume in the various groups were measured (Fig. 2B). In mice receiving one administration of radiation at 8 Gy with control IgG (RT), mean tumor volumes were reduced at 15 days when compared to controls (CON; P < 0.05). Tumors treated with rhuMAb HER-2 alone (MAb) showed less growth reduction, but combined radiation-antibody therapy produced marked reduction in tumor volumes over the 15-day treatment period when compared to control or either treatment alone (P < 0.001). These data, in combination with the results in Fig. 2A, show superior efficacy of radiation when given with rhuMAb HER-2 and demonstrate a clear therapeutic advantage with this treatment regimen.

Anti-HER-2 Antibodies Block DNA Repair in Response to Ionizing Radiation in Human Breast Cancer Cells. After demonstrating a therapeutic advantage for the combination of antibody and radiation in HER-2 overexpressing cells, studies were designed to evaluate the possible mechanism(s) for this phenomenon. Studies have shown that DNA repair plays an important role in the recovery of cells from the toxicity of ionizing radiation (41). Prior work has also shown that inhibition of DNA repair by anti-HER-2 receptor antibodies is important in antibody-enhanced cytotoxicity of cisplatin in HER-2-overexpressing breast and ovarian cancer cells (22). To evaluate whether similar alterations in DNA repair may be a potential explanation for the enhanced effects of antireceptor antibody and radiation, we measured DNA repair induced by radiation in SKBR3 and MCF-7/HER-2 cells using autoradiographic localization of [3H]thymidine over cell nuclei (36) to provide a quantitative measure of this phenomenon. As expected, radiation exposure induces enhanced UDS in SKBR3 cells (Fig. 3). Exposure to rhuMAb HER-2 alone has no such effects on these cells. The radiation-induced effect, however, was blocked by pretreatment of the cells with antireceptor antibody (Fig. 3). To determine whether this phenomenon was restricted to a specific cell line and to study its association with HER-2 overexpression, we performed similar studies in MCF-7 and MCF-7/HER-2 cells. These two cell lines are identical to one another except for the presence of HER-2 overexpression in the MCF-7/HER-2 cells. Radiation elicited a marked increase in DNA repair in the PAR, CON, and HER-2 cells. However, this radiation-induced effect was blocked by rhuMAb HER-2 in the MCF-7/HER-2 cells specifically and did not occur in control cells. These data confirm that rhuMAb HER-2 interferes with DNA repair only in those cells overexpressing the HER-2 receptor (Fig. 3). Using an alternative measure of DNA repair, we observed the same phenomenon with a transfected CMV-driven β-galactosidase reporter plasmid (36). At 24 h posttransfection, the extent of repair was assayed by measuring reporter DNA expression in MCF-7/HER-2 cells that were either incubated with rhuMAb HER-2 or control media after transfection (Fig. 4). The transfected cells were then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, a substrate for β-galactosidase, to distinguish β-galactosidase-positive and -negative cells. In the presence of substrate, cells expressing the reporter bacterial β-galactosidase protein appear blue and the percentage of stained cells can be quantitated. These data show that antibody treatment elicits blockade of repair of the radiation-damaged reporter DNA (Fig. 4), again demonstrating a therapeutic advantage seen in cells overexpressing HER-2 after treatment with a combination of antibodies to the HER-2 receptor and radiation.
Presented as the percentage of blue-stained cells in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, a substrate for β-galactosidase.

**Influence of Antireceptor Antibodies on Cell Cycle Regulation in Human Breast Cancer Cells.** To further characterize effects of rhuMAb HER-2 on breast cancer cell growth, analyses of cell cycle phase distribution were conducted. Previous reports have shown that the 4D5 antibody directed against the HER-2 receptor reduces the fraction of HER-2-overexpressing cells in S phase (34). Using a similar approach, we grew MCF-7/HER-2 cells in serum-depleted medium and then treated them with 0 or 10% serum-enriched medium. The cells were then exposed to 4 Gy of radiation with or without prior exposure to rhuMAb HER-2. Results of these studies demonstrate that maintenance of cells in serum-depleted medium reduces the fraction of cells in S-phase compared to control cells in medium with 10% serum (Fig. 5). Treatment with rhuMAb HER-2 reduces the fraction of MCF-7 HER-2 cells in S phase at both 24 and 48 h. Radiation treatment of the cells also promotes cell cycle arrest, predominantly at G1, again resulting in a low S-phase fraction at 24 and 48 h. In the presence of rhuMAb HER-2, radiation elicits a similar reduction in S phase at 24 h; however, a significant reversal of the cell cycle arrest appears by 48 h postradiation (P < 0.001; Fig. 5). Unlike the non-antibody-treated cells, the S-phase fraction of these cells is significantly greater at 48 h compared to the fraction at 24 h. These results indicate that early escape from cell cycle arrest in the presence of antireceptor antibody may not allow sufficient time for completion of DNA repair in HER-2-overexpressing cells.

**Influence of Antireceptor Antibodies on Regulation of p21WAF1 Transcript Levels, Protein Levels, and Phosphorylation State.** To further explore the molecular basis for these observations, we performed a series of studies evaluating the p21WAF1 activity in these cells. The tumor suppressor protein p53 is known to be a critical mediator of the cellular response to DNA damage (25, 27). Induction of the CDK inhibitor, p21WAF1, in response to DNA damage occurs primarily through a transcriptional mechanism that requires interaction of the p53 protein with a p53-binding site in the p21WAF1 promoter (28). Recent studies, however, have shown that induction of p21WAF1 following growth factor stimulation may not always require p53 and may, instead, be directly activated by MAP kinase (31). Consistent with this is the observation that withdrawal of growth factors in vitro is associated with down-regulation of p21WAF1 expression and with enhanced cell killing in response to DNA damage (3). It is known that basal levels of p21WAF1 are not sufficient to cause cell cycle arrest and that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest. This results in a reduced time for completion of DNA repair. To assess the activity of p21WAF1 in response to DNA damage in MCF-7/HER-2 cells in the presence or absence of the anti-HER-2 antibody, we first performed Northern blot analyses of p21WAF1 expression levels. MCF-7/HER-2 cells were treated with rhuMAb HER-2 alone or prior to radiation exposure to 6 Gy. Parallel cells were treated with either control solution alone or radiation alone. At 6, 12, and 24 h, cells were processed for RNA extraction and determination of p21WAF1 transcript levels. As expected, progressive induction of p21WAF1 transcripts was seen at 6–24 h post-radiation treatment (Fig. 6A); however, increased levels of p21WAF1 transcript were not sustained in MCF-7 HER-2 cells that had been exposed to radiation in the presence of rhuMAb HER-2. Although p21WAF1 transcript level increases at 6–12 h, it is comparable to baseline levels by 24 h (Fig. 6A). Moreover, the level of p21WAF1 at 24 h is markedly less than the levels seen after radiation given without antibody. A clear reduction in the basal level of p21WAF1 also occurred after 12–24 h exposure to antibody alone, compared to controls. In contrast, the level of p53 transcripts was only slightly increased by radiation after 6 h or 24 h, and no attenuation of the transcript level occurred after combined treatment with rhuMAb HER-2 (Fig. 7A). An additional transcript, cyclin D1, showed no variation with antibody, radiation or combination therapy (Fig. 7B).

Western analyses of the level of p21WAF1 protein in MCF-7/HER-2 cells likewise show enhanced amounts of the protein at 6–24 h after radiation (Fig. 6B); however, consistent with the Northern blot studies, treatment of cells with antireceptor antibody elicits a reduced level of p21WAF1 protein under basal conditions and blunts the anticipated response to radiation therapy at 12–24 h, as compared to controls. Radiation with 6 or 10 Gy elicits a significant increase in the level of mdm2 protein, whereas a pronounced decrease in mdm2 protein level occurs when radiation is administered in the presence of anti-HER-2 receptor antibody (Fig. 8A). Similarly, the level of p53 protein increases in response to radiation after 6 and 24 h, and, as with p21WAF1, there is a slight reduction in the expected response to radiation when cells are treated concomitantly with rhuMAb HER-2 (Fig. 8B).
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Fig. 6. Monoclonal antibody to HER-2 receptor alters p21WAF1 transcript and protein levels and the tyrosine phosphorylation of p21WAF1 after radiation treatment of human breast cancer cells. A, MCF-7 HER-2 cells were treated with control solution (Lane Cn), 200 μg/ml rhuMAb HER-2 alone (Lane Ab), 6 Gy radiation alone (Lane RT), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (Lane Ab/RT). After 6, 12, or 24 h, cells were processed for preparation of RNA and determination of p21WAF1 transcripts using Northern blot. B, MCF-7 HER-2 cells were treated with control solution (Lane Cn), 200 μg/ml rhuMAb HER-2 alone (Lane Ab), 6 Gy radiation alone (Lane RT), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (Lane Ab/RT). After 6, 12, or 24 h, cells were processed for Western blot analysis and determination of the level of tyrosine phosphorylation of p21WAF1 by first immunoprecipitating with antiphosphotyrosine antibody and then immunoblotting with anti-p21WAF1 antibody. C, MCF-7 HER-2 cells were treated with control solution (Lane Cn), 200 μg/ml rhuMAb HER-2 alone (Lane Ab), 6 Gy radiation alone (Lane RT), or 200 μg/ml rhuMAb HER-2 in combination with 6 Gy radiation (Lane Ab/RT). After 6, 12, or 24 h, cells were processed for Western blot analysis and determination of the level of tyrosine phosphorylation of p21WAF1 by first immunoprecipitating with antiphosphotyrosine antibody and then immunoblotting with antiphosphotyrosine antibody. See text for additional details.

In view of the crucial role of tyrosine phosphorylation in regulating the activity of diverse signaling molecules (4, 8, 9), we assessed the potential influence of the HER-2 receptor pathway on phosphorylation of tyrosine residues in p21WAF1 (28). We first tested whether p21WAF1 is a substrate for tyrosine phosphorylation induced by radiation. A number of protein kinases are known to be induced by stress and/or DNA damage and are hypothesized to play a role in DNA repair by phosphorylating regulatory proteins (30). MCF-7/HER-2 cells show little to no tyrosine phosphorylation of p21WAF1 under basal conditions (Fig. 6C); however, radiation exposure elicits p21WAF1 tyrosine phosphorylation, which is evident after 6 and 12 h and dissipates by 24 h (Fig. 6C). In the presence of anti-HER-2 receptor antibody, radiation-induced tyrosine phosphorylation of p21WAF1 occurs by 6 h, but the phosphorylation is not sustained and returns to baseline levels by 12 h (Fig. 6C). These results suggest that dysregulation of radiation-induced p21WAF1 tyrosine phosphorylation occurs after treatment with antireceptor antibody, and this event may adversely influence the cell response to DNA damage.

DISCUSSION

The potential role of HER-2 and related erb B proto-oncogenes in the modulation of sensitivity to radiation has been suggested from some studies (20, 21). Transformation of NIH-3T3 cells with HER-2 cDNA from esophageal carcinoma leads to induction of radiation resistance (20). Here, we used matched MCF-7 parent and daughter human breast cancer cells, which differ in their HER-2 expression level, to evaluate effects on radiation sensitivity. These paired cells were used to circumvent the possibility that phenomena due to effects other than HER-2 overexpression (i.e., cell line specific), might be observed. In addition, we used the naturally overexpressing cell line SKBR3 to ensure that the results were not restricted to a single cell line and were not due solely to artificial engineering of the cells. Using this approach, we were able to directly compare in vitro radiation sensitivities of parental cells with low-expression of HER-2 to identical daughter cells with high-expression of HER-2. The data demonstrate that the D_{10} of HER-2-overexpressing breast cancer cells is increased by ~25% compared to cells with the normal complement of HER-2 receptors. These findings are consistent with independent
studies in which antisense oligonucleotides directed against the HER-2 gene were able to reverse the radiation resistance of human tumor cell lines with HER-2 overexpression (42). These analyses suggest a potential biological role for the HER-2 oncogene in resistance to radiation treatment and may have important implications in the clinical management of patients whose breast cancers contain this alteration. To evaluate the therapeutic advantage of combined treatment with radiation and antibodies to HER-2, we conducted a series of in vitro studies, which show that anti-HER-2 receptor antibody enhances radiation-induced killing of naturally overexpressing SKBR3 human breast cancer cells as well as MCF-7 cells engineered to overexpress this receptor. Further tests demonstrate a significant growth-inhibitory therapeutic advantage of ionizing radiation combined with rhuMAb HER-2 in HER-2 overexpressing human breast cancer xenografts in nude mice. Mice receiving radiation alone do not have significantly reduced tumor volumes over the 7-week observation period, and no tumor remissions were observed. Treatment with rhuMAb HER-2 alone similarly elicited no significant tumor remissions. In contrast, the combination of radiation and antibody therapy produced marked reductions in tumor growth, and all animals receiving both rhuMAb HER-2 and radiation show complete tumor remissions, demonstrating a marked in vivo enhancement of radiation efficacy when given with anti-HER-2 antibody in HER-2-overexpressing breast cancers.

A spectrum of lesions is known to be induced in DNA by radiation, and DNA repair plays an important role in the recovery of cells from the toxicity of radiation exposure (41). Changes in DNA repair have been reported to occur in HER-2-overexpressing cells after treatment with antibodies to HER-2 receptor (22). To further evaluate the possible role of DNA repair as an explanation for the therapeutic advantage of anti-HER-2 antibody combined with DNA-damaging radiation, we measured DNA repair, cell cycle regulation and selected molecular alterations induced by radiation in breast cancer. These studies show that radiation enhances UDS, a measure of DNA repair, in human breast cancer cells with HER-2 overexpression. This radiation-induced effect is blocked by treatment of the cells with anti-HER-2 antibody. Additional tests of DNA repair using a CMV-driven β-galactosidase reporter plasmid exposed to radiation in vitro demonstrate that repair of radiation-damaged DNA proceeds in the absence of rhuMAb HER-2 but is significantly reduced when the antibody is administered to human breast cancer cells containing the HER-2 alteration. Moreover, this phenomenon is specific to cells overexpressing HER-2.

Prior studies have shown that the 4D5 antibody reduces the fraction of HER-2-overexpressing cells in S phase (33). Conducting additional studies of cell cycle phase distribution here, we demonstrated that rhuMAb HER-2 reduced the fraction of MCF-7 HER-2 cells in S phase at both 24 and 48 h (33). This study also indicates that radiation promotes cell cycle arrest predominantly at G1, with a low S-phase fraction observed at 24 and 48 h. In the presence of rhuMAb HER-2, radiation elicited a similar reduction in S-phase at the early time point, i.e., 24 h, but a significant reversal of cell cycle arrest occurred at 48 h postradiation exposure. Hence, early escape from cell cycle arrest in the presence of anti-HER-2 antibody may not allow sufficient time for completion of DNA repair in HER-2-overexpressing cells, resulting in accumulation of DNA damage and greater cell death.

The tumor suppressor gene product p53 is a critical mediator of the cellular response to DNA damage. Although induction of the CDK inhibitor, p21WAF1, in response to DNA damage occurs primarily through a transcriptional mechanism involving p53 (28), some reports suggest that p21WAF1 may enhance the radiosensitivity of tumor cells independent of p53 (31, 43). In p21WAF1−/− colon cancer cells, p21WAF1 deficiency is associated with a prominent defect in DNA repair (37). Recent work suggests that certain growth factors interacting with their respective receptors may provide an alternate pathway for regulation of p21WAF1 expression. Activation of some growth factor stimulatory pathways results in induction of p21WAF1, which does not require p53 and may, instead, be activated by MAP kinase (31). In addition, withdrawal of growth factors in vitro has been associated with down-regulation of p21WAF1 expression and
enhanced cell killing in response to DNA damage. This study provides further evidence that the growth factor receptor, HER-2, can modulate DNA damage response pathways in human breast cancer cells and suggests that this cross-communication may involve modulation of p21WAF1. A notable reduction in the basal level of p21WAF1 occurred after exposure to anti-HER-2 receptor antibody when compared with controls, indicating that interactions with the HER-2 pathway can directly affect p21WAF1 expression. These results and independent reports on depletion of p21WAF1 after withdrawal of growth factors (3, 44) suggest an important role for at least some growth factor receptor pathways in modulating the activity of proteins that regulate the cell cycle in response to DNA damage. It is well known that basal levels of p21WAF1 are not sufficient to cause cell cycle arrest and that failure of adequate p21WAF1 induction after DNA damage is associated with failure of cell cycle arrest, resulting in reduced time for completion of DNA repair. After radiation, the expression of p21WAF1 is increased in MCF-7/HER-2 cells, but the increased levels of p21WAF1 transcripts and protein are not sustained in HER-2-overexpressing cells exposed to radiation in the presence of rhuMAb HER-2. In contrast, levels of p53 transcript and protein were only slightly increased by radiation, and only minimal reductions occurred on combined treatment with rhuMAb HER-2. This suggests that induction of p21WAF1 in HER-2-overexpressing MCF-7 cells may be less dependent on regulation by p53 and may involve alternative signal transduction pathways (31, 42, 44).

In view of the crucial role of phosphorylation in regulating the activity of diverse signaling molecules (4, 8, 9), we assessed the potential influence of the HER-2 receptor signaling pathway and radiation on phosphorylation of tyrosine residues in p21WAF1 (28). A number of protein kinases are well known to be induced by stress and/or DNA damage and are hypothesized to play a role in DNA repair by phosphorylation of regulatory proteins (30). Although MCF-7/HER-2 cells show little to no tyrosine phosphorylation of p21WAF1 under basal conditions, radiation treatment induces a transient tyrosine phosphorylation of p21WAF1, an effect that is clearly diminished by anti-HER-2 receptor antibody. These results suggest that dysregulation of radiation-induced p21WAF1 tyrosine phosphorylation occurs after treatment with antireceptor antibody. The potential outcome of this molecular alteration on the biological activity of p21WAF1 remains to be determined. However, independent studies show that DNA damage promotes specific alterations in the phosphorylation state of other DNA-regulatory factors, such as p53 (45) and BRCA1 (46), leading to changes in nuclear localization and in specific molecular interactions. Tyrosine residues of p21WAF1 occur in functional domains known to be associated with nuclear localization and with binding to cyclin/CDKs and to PCNA (28, 47). Moreover, the tyrosine residues in p21WAF1 of human and mouse origin are highly conserved in corresponding regions of other human CDK inhibitors, p27Kip1 and p57Kip2 (47), suggesting that these proteins may share a similar mechanism of action. It will be important to direct future studies to investigation of the role of tyrosine phosphorylation of p21WAF1 in critical molecular interactions.

Significant data support the hypothesis that p21WAF1 may play a vital role in mediating the rhuMAb HER-2 effects on DNA damage pathways in HER-2-overexpressing breast cancer cells. DNA replication and repair may be coordinated by differential effects of p21WAF1 on replicative and repair DNA synthesis. The p21WAF1 protein interacts with CDKs and PCNA, a protein important for regulation of both DNA replication and repair processes (3, 31, 48). This work suggests that alterations in other regulatory proteins, such as mdm2 (29), may also contribute to this process. Radiation elicits a significant rise in the level of mdm2 protein that is sustained over several hours. However, when radiation is administered in the presence of anti-HER-2 receptor antibody, the initial increment in the level of mdm2 protein is not maintained, with a pronounced decline in mdm2 by 24 h. These changes in mdm2 levels may be important because the oncoprotein contains inhibitory domains that can interfere with both p53-dependent (49) and -independent (50) transcriptional activity. Promotion of apoptosis (3) by rhuMAb HER-2 could be another postulated outcome of treatment, but we have been unable to document alterations in bcl-2 protein or DNA fragmentation in MCF-7/HER-2 cells after low, sublethal doses of ionizing radiation with or without anti-HER-2 antibody (51). Nevertheless, the available evidence suggests that pathways of DNA replication, DNA repair and DNA degradation may have common regulatory elements, with the final cellular outcome being dependent on the extent of DNA damage (48).

Future work will be required to fully understand how a MAP kinase may play a role in the regulation of p21WAF1 through growth factor receptors. Heregulin, a natural ligand to HER-2/HER-3 heterodimers, induces transient phosphorylation of HER-2 protein, promoting downstream activation of MAP kinase (38, 52). In contrast, antibodies to the HER-2 receptor induce prolonged phosphorylation and downregulation of HER-2 protein and disrupt the association of HER-2 with HER-3 (38, 52–54). Similar to anti-HER-2 antibodies, tyrosine kinase inhibitors with specificity for the HER-2 kinase are also known to enhance the sensitivity of HER-2-overexpressing cancer cells to DNA-damaging agents (55). Although the activity of the anti-HER-2 antibody remains to be fully characterized, downstream effects of HER-2 stimulation, such as activation of MAP kinase and other protein kinases, are likely to be affected by rhuMAb HER-2.

This study suggests that human breast cancer cells damaged by radiation may be especially vulnerable to injury if they are also deprived of essential signal transduction mechanisms by disruption of the HER-2 growth factor receptor pathway. Growth factor receptors appear to play a significant role in the regulation of cell cycle checkpoints and repair of DNA damage, and manipulation of this pathway in the clinic using rhuMAb HER-2 may provide therapeutic benefit to patients with HER-2-overexpressing breast cancers.

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