Radiation-induced Dimer Formation of EGFR: Implications for the Radiosensitizing Effect of Cetuximab

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Abstract. *Aim: The purpose of this study was to investigate whether radiation induces ligand-independent dimerization of epidermal growth factor receptor (EGFR) and explore the possible role of radiation-induced receptor dimerization in the radiosensitizing effect of cetuximab. Materials and Methods: The human vulvar squamous cell carcinoma cell line A431 was used. The dimerization and activation of EGFR were quantified using immunoprecipitation, a western blotting analysis, and a chemical cross-linking analysis with dithiobis-sulfosuccinimidyl propionate. Results: Irradiation at a dose of 2 Gy induced the autophosphorylation of EGFR. Consistent with autophosphorylation, a 360-kDa polypeptide, corresponding to the size of the EGFR dimer, was detected in addition to an EGFR monomer. Radiation also induced hetero-dimerization between EGFR and HER2/neu. Cetuximab combined with radiation inhibited radiation-induced autophosphorylation of EGFR, and inhibited radiation-induced homo-dimerization of EGFR. However, cetuximab incompletely inhibited radiation-induced hetero-dimerization between EGFR and HER2. Conclusion: The results of this investigation suggest that radiation-induced homo- and/or hetero-dimerization between EGFR and/or HER2 might be involved in the radioresponse of cancer cells.*

Molecules and genes that are involved in the determination of the radiosensitivity of cancer cells have been reported in numerous studies. Nevertheless, the factors that determine radiosensitivity have not been fully-elucidated, and numerous tumors are radiation-resistant and difficult to control despite the use of high-dose radiation therapy with and without chemotherapy. Recent investigations regarding the cellular responses to radiation have revealed that radiation activates various signal transduction pathways *via* the induction of genes that encode transcription factors or *via* the direct activation of kinases (1, 2). This results in the alteration of the expression or activation of certain receptors on the cell surface or of the subcellular distribution of proteins and molecules that transmit signals to the nucleus. These responses include molecules and related signal transduction pathways that are associated with a cytoprotective response (3). In particular, the impact of receptor tyrosine kinase receptors (RTK), including epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF), and HER2/neu, and their downstream signal transduction pathways on the cellular response to radiation has been intensively investigated (4-6). We have reported on an inverse correlation between EGFR expression and radiosensitivity of murine tumors, indicating that tumors with high EGFR expression tend to be more radioresistant than tumors with low expression (7). We demonstrated that radiation-induced autophosphorylation of EGFR and the activation of downstream signals were involved in the mechanism underlying the acquisition of a radioresistant profile, but only in tumors with high levels of EGFR expression.

An effective approach to enhancing or sensitizing cells to radiation-induced cell killing is to inhibit or suppress the activity of molecules or signal transduction pathways that are responsible for radioresistance. In order to target RTKs and related signal transduction pathways, cetuximab (C225; a human mouse chimeric monoclonal antibody for EGFR), ZD1839, erlotinib (a tyrosine kinase inhibitor for EGFR), and Herceptin (a monoclonal antibody for HER2/neu) alone or in combination with radiation therapy have already been used in clinical trials as single-agents. In terms of pre-clinical studies, Huang *et al*. demonstrated that C225 combined with radiation enhanced radiosensitivity and amplified radiation-induced apoptosis

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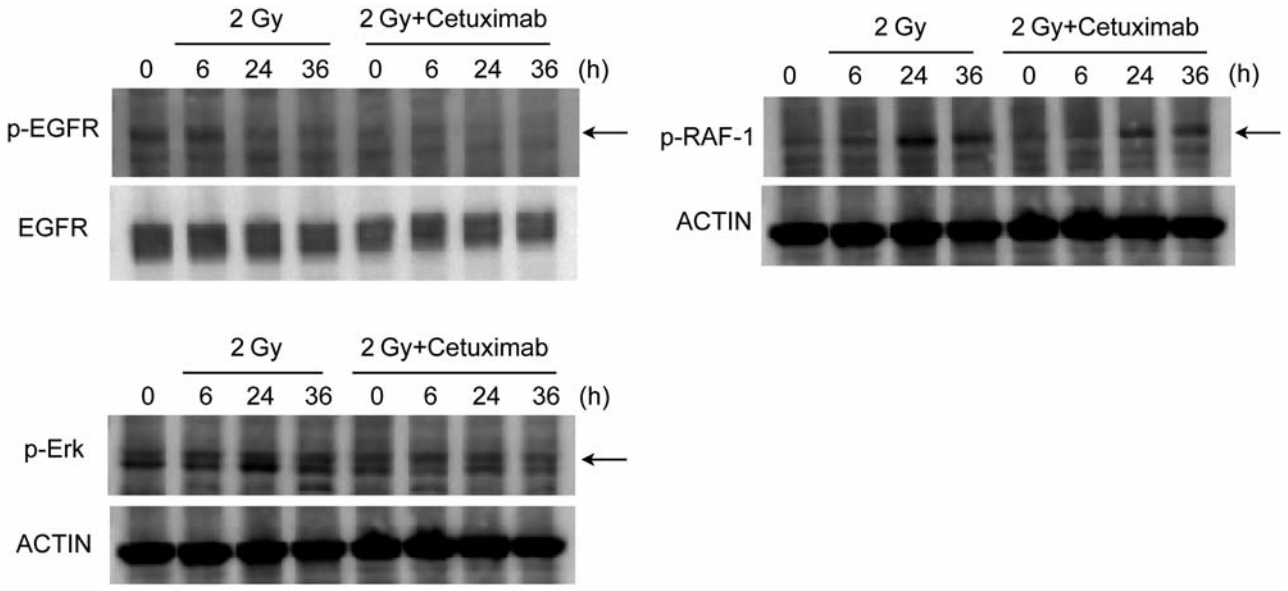


Figure 1. *Cetuximab combined with radiation inhibited the radiation-induced activation of EGFR and its signal transduction. Cells were exposed to radiation alone at a dose of 2 Gy or were exposed to radiation in the presence of cetuximab of 10 μg/ml. The cells were irradiated 24 h after the start of incubation with cetuximab, and samples for western blotting were obtained at the indicated time-points after irradiation.*

in squamous cell carcinoma (SCC) cell lines derived from head and neck cancer (8). Milas *et al*. also reported that C225 in combination with radiation resulted in a synergistic growth delay in EGFR-overexpressing A431 tumors in nude mice (9). Schmidt-Ullrich *et al*. reported that ionizing radiation induces EGFR autophosphorylation, thereby activating the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI3-K) /Akt pathways and increasing the proliferation of A431 human squamous carcinoma cells (10). Therefore, radiation-induced autophosphorylation is a critical event in the mechanism underlying the modification of the radiation response through EGFR and its signal transduction pathways. Ligand binding is known to induce the homo- and/or hetero-dimerization of ERBB receptors including EGFR, resulting in the phosphorylation of tyrosine residues in the C-terminal tail (11, 12). Auto-phosphorylation involves the phosphorylation of intracellular tyrosine residues; hence, it is important to investigate whether the dimerization of the receptor precedes radiation-induced autophosphorylation. However, whether radiation exposure induces the dimerization of EGFR in a manner similar to ligand binding has not been fully- elucidated. The purpose of this study was to examine whether radiation induces ligand-independent EGFR dimerization and to investigate the possible role of radiation-induced receptor homo- and/or hetero-dimerization in the radiosensitizing effect of cetuximab.

Materials and Methods

Cell culture. The human vulvar squamous cell carcinoma cell line A431, which exhibits a high level of EGFR expression, was used in this study. The cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum at 37˚C.

Reagents and antibodies. Cetuximab, a mouse-human chimeric monoclonal antibody for EGFR, was kindly provided by Merck Co. Ltd, Darmstadt, Germany. Mouse monoclonal antibody (mAb) against EGFR (1F4), mouse anti-phospho-EGFR (Tyr1068) (1H12) mAb, mouse anti-HER2/ERBB (44E7) mAb, rabbit-phospho-HER2/ERBB (Tyr1248) antibody, mouse anti-ERK mAb, and mouse anti-RAF-1 mAb were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). The ECL chemiluminescence detection system was purchased from Amersham (Arlington Heights, IL, USA), and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Irradiation method and clonogenic survival assay. Radiosensitivity was determined using a clonogenic assay. Trypsinized cells were seeded for a colony formation assay in 60-mm diameter culture dishes containing 5 mL of medium. The plated cells were then irradiated with 150-kV photons (MBR-1505R2; Hitachi Medico, Tokyo, Japan) at a dose rate of 1.32 Gy/min at room temperature. The colonies were fixed and stained with crystal violet (2% in methanol) at least 14 days after subculturing for counting. To determine the combined effect of cetuximab on radiosensitivity, cetuximab was incubated with the cells for 72 h at a concentration of 10 μg/ml, and the cells were irradiated at 24 h after the start of incubation with cetuximab. Exponentially-growing cells were used for each experiment, and the experiments were repeated five times.

Chemical cross-linking. To examine whether radiation induced EGFR dimerization, chemical cross-linking was performed. After treating the cells with irradiation at a dose of 2 Gy or epidermal growth factor (EGF) at a concentration of 100 ng/mL for 3 min, samples for the chemical cross-linking analysis were obtained immediately after irradiation and at 30 min after irradiation. The cells were washed with Phosphate buffered saline (PBS; 137 mM NaCl, 0.67 mM KCl, 8 mM Na_2HPO_4, and 1.4 mM KH_2PO_4) three times and incubated for 30 min at 4˚C with 1 mM 3,3′-dithio*bis*-[sulfosuccinimidylpropionate] (DTSSP; Pierce Chemical Co., Rockford, IL, USA) in PBS, followed by washing three times with Tris-buffered saline [TBS; 20 mM Tris-HCl, 100 mM NaCl (pH 7.5)] in the following studies.

Western blotting. To examine the changes in the phosphorylation of EGFR and other molecules, a western blot analysis was performed. To investigate irradiation-induced EGFR dimerization, a western blot using a cross-linking analysis with DTSSP was undertaken. After irradiation followed by cross-linking with DTSSP, the cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% TritonX-100, 2 mM, ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% phosphatase inhibitor cocktail 1 (Sigma Chemical Co.), and 1% phosphatase inhibitor cocktail 2 (Sigma Chemical Co.) The cell suspension was centrifuged for 12 min at 15000 × *g*. The samples were boiled for 10 min in SDS-polyaclylamide gel electrophoresis(SDS-PAGE) sample buffer without β-mercaptoethanol, and equal amounts of protein were run on SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% skim milk in PBS, then incubated with primary antibody in PBS containing 0.1% Tween20 for 1 h at room temperature or overnight at 4˚C. Next, the membrane was incubated with Horse Radish Peroxidase (HRP)-conjugated species-specific secondary antibody at room temperature for 30 min and detected using the ECL western blotting detection system (Amersham International, Little Chalfont, UK). The total amount of cellular proteins applied to each lane was equalized using BCA protein assay reagent (Pierce, Rockford, IL, USA). The intensity of the bands was quantified using the National Institutes of Health Image J.

Immunoprecipitation analysis. To investigate the changes in EGFR–EGFR homo-dimer, HER2–HER2-homo-dimer, and EGFR–HER2 hetero-dimer formation caused by irradiation with or without cetuximab, an immunoprecipitation analysis was performed using a commercially available immunoprecipitation kit (Millipore, Massachusetts, MA, USA). The procedure was based on the manufacturer's instructions as follows. The cells were lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% TritonX-100, 2 mM ethylenediaminetetra-acetic acid, 1 mM PMSF, 1% phosphatase inhibitor cocktail 1 (Sigma Chemical Co.), and 1% phosphatase inhibitor cocktail 2 (Sigma Chemical Co.). The lysates were cleared by centrifugation and immunoprecipitated with antibody against EGFR for 30 min at room temperature. The collected proteins were subjected to western blot analysis as described above using an antibody to EGFR or HER2.

Statistics. Differences between groups were analyzed using an unpaired two-tailed *t*-test. A *p*-value <0.05 was considered statistically significant.

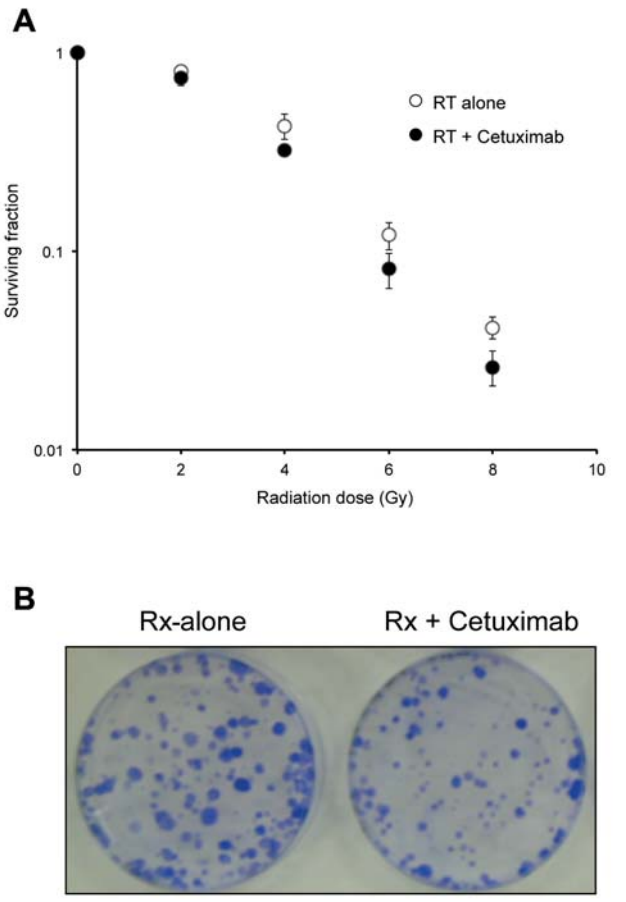


Figure 2. *Synergistic enhancement of radiosensitivity of A431 cell caused by cetuximab. A: Cell survival curve after irradiation with or without cetuximab. Cellular radiosensitivity was determined using a clonogenic assay. Cells were irradiated in the presence or absence of cetuximab at the indicated dose, and the colonies were stained 14 days after irradiation. To examine the effect of cetuximab on cellular radiosensitivity, cells were incubated in the presence of cetuximab at a concentration of 10 μg/ml for 72 h. Cells were irradiated 24 h after the start of incubation with cetuximab, and cetuximab was washed out 48 h after irradiation. Vertical bars represent the standard deviation. B: Colony formation. Colonies were stained after irradiation with 4 Gy with and without cetuximab treatment of 10 μg/ml. C: The number of colonies after irradiation combined with cetuximab treatment was smaller than the one after irradiation alone, although the same number of trypsinized cells was seeded. In addition, the size of the colonies after irradiation combined with cetuximab treatment was slightly smaller than the one after irradiation alone.*

Results

Effects of radiation on phosphorylation and activation of EGFR and its downstream molecules. Figure 1 shows the results of a western blot examining changes in the phosphorylation of EGFR, RAF-1 and ERK after irradiation at a dose of 2 Gy with and without cetuximab treatment at a

concentration of 10 μg/ml. Irradiation at a dose of 2 Gy increased the phosphorylation of EGFR, indicating that irradiation induced the autophosphorylation of EGFR. Consistent with EGFR phosphorylation, the downstream molecules RAF-1 and ERK were also phosphorylated upon cell irradiation. Cetuximab at a concentration of 10 μg/ml combined with radiation abolished the radiation-induced phosphorylation of EGFR; however, the increase in RAF-1 and ERK phosphorylation was only marginally-inhibited by cetuximab.

Effect of cetuximab on radiosensitivity. The effect of cetuximab in combination with radiation on radiosensitivity was examined using a clonogenic assay. Figure 2A shows that radiation in combination with cetuximab at a concentration of 10 μg/ml for 72 h, synergistically enhanced the radiosensitivity of A431 cells. The surviving fraction of cells after cetuximab treatment alone at a concentration of 10 μg/ml for 72 h was 0.42±0.03 when the surviving fraction of the control (no treatment) was assumed to be 1 and the survival curves were normalized for cetuximab-induced cell death. The difference in the surviving fractions after irradiation at 4, 6 and 8 Gy between the irradiation-alone and the irradiation combined with cetuximab groups was statistically significant. As shown in Figure 2B, cetuximab combined with radiation reduced the size of the colonies, in addition to the number of colonies formed, compared with the irradiation-alone group.

Radiation-induced dimer formation of EGFR and HER2. Western blotting analysis using a chemical cross-linking analysis was used to investigate whether radiation induces EGFR homo-dimerization in addition to the autophosphorylation of EGFR. As shown in Figure 3, a 360-kDa polypeptide corresponding to the size of the EGFR dimer was detected in addition to the 180-kDa EFGR monomer by applying an antibody to EGFR antibody to EGF-stimulated cells (100 ng/ml for 3 min) treated with DTSSP. To detect radiation-induced EGFR dimerization, cells were irradiated at a dose of 2 Gy in the presence of DTSSP and samples were obtained immediately after irradiation and 30 min after irradiation. Irradiation at a dose of 2 Gy induced a 360-kDa polypeptide, corresponding to the size of the EGFR dimer, in addition to the 180-kDa EGFR monomer similar to the results obtained in EGF-stimulated cells.

To examine the effect of radiation with and without cetuximab treatment on EGFR–EGFR homo-dimer, HER2–HER2 homo-dimer, or EGFR–HER2 hetero-dimer formation, an immunoprecipitation analysis was performed as described in the Materials and Methods section. Figure 4A shows the results for a cell lysate immunoprecipitated using the antibody to EGFR, indicating that radiation induced EFGR homo-dimerization similar to that obtained after stimulation with EGF at a concentration of 100 ng/ml for 3 min. The level of hetero-dimerization between EGFR and HER2 did not increase after radiation; however, whether the radiation actually induced hetero-dimerization was unclear because as seen in Figure 4A we only obtained results at a single time-point, immediately after irradiation. Therefore, as shown in Figure 4B, the post-irradiation changes in dimer formation were also examined. Cell lysate immunoprecipitated using antibody against HER2 was used for this analysis. The upper bands in Figure 4B indicate that irradiation at a dose of 2 Gy did not cause any noticeable changes in the homo-dimerization of HER2, compared with no treatment; however, hetero-dimerization between EGFR and HER2 was augmented at 10 min after 2 Gy of irradiation (middle bands in Figure 4B). The lower bands in Figure 4B show that irradiation also induced an increase in the hetero-dimerization between HER2 and phosphorylated EGFR soon after irradiation. These findings demonstrated that radiation induced hetero-dimerization between EGFR and HER2 to a greater extent than that observed in the steady state, in addition to the homo-dimerization of EGFR, and the fact that association of HER with phosphorylated EGFR preceded hetero-dimerization between EGFR and HER2.

Effects of cetuximab on radiation-induced dimerization and activation of EGFR and HER2. The effect of cetuximab on the irradiation-induced homo-dimerization of EGFR and hetero-dimerization of EGFR and HER2 was investigated to explore the role of the changes in dimer formation on the underlying mechanism responsible for the radiosensitizing effect of cetuximab. An immunoprecipitation analysis was also used for this analysis. Figure 5A shows the results obtained using cell lysate immunoprecipitated by an antibody against EGFR. As shown in Figure 5A, irradiation at a dose of 2 Gy induced the homo-dimerization of EGFR. The right two bands of Figure 5A represent the results after cetuximab treatment with and without irradiation. The intensities of the bands after cetuximab treatment with and without irradiation were stronger than those for the control and after irradiation alone. This result might be attributable to the fact that the resultant lysate immunoprecipitated by an antibody against EGFR included EGFR-cetuximab complexes in addition to cetuximab-unbound. The intensity of the band after irradiation at a dose of 2 Gy combined with cetuximab was 0.9 when the intensity of the band after cetuximab alone was assumed to be 1, indicating that the intensity of both bands was almost the same. This finding would be indirect evidence for inhibition of the radiation-induced homo-dimerization of EGFR by cetuximab. Figure 5B shows the changes in the formation of hetero-dimerization between EGFR and HER2 after radiation alone and after radiation combined with cetuximab treatment. As shown in Figure 4B, radiation increased the level of hetero-

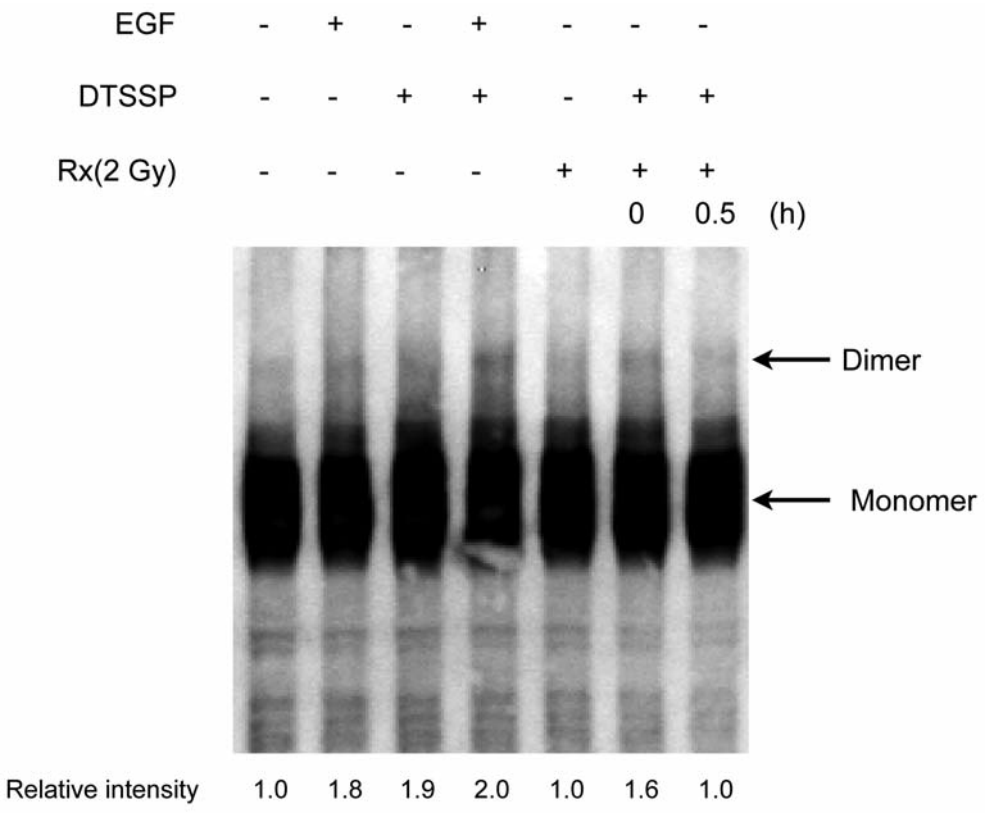


Figure 3. *Radiation-induced epidermal growth factor receptor (EGFR) dimer formation. After irradiation at a dose of 2 Gy or epidermal growth factor (EGF) stimulation at a concentration of 100 ng/ml for 3 min followed by cross-linking with dithiobis-sulfosuccinimidyl propionate (DTSSP), samples were obtained at the indicated time-points. Cells were lysed as described in the Materials and Methods section, and equal amounts of proteins were run on SDS-polyaclylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk, the membrane was incubated with antibody to EGFR for 1 h at room temperature, then incubated with Horse Radish Peroxidase (HRP)-conjugated secondary antibody at room temperature for 30 min. A western blotting detection system was used for chemiluminescence detection.*

dimerization between EGFR and HER2 at 30 min and 3 h after irradiation, and cetuximab treatment in combination with irradiation maintained hetero-dimerization at a level almost equal to the one observed in the steady state. This result suggested that cetuximab also inhibited the radiation-induced hetero-dimerization of EGFR and HER2. However, the results of immunoprecipitation using the cell lysate immunoprecipitated by an antibody to HER2, demonstrated that the intensity of the bands after cetuximab treatment with and without irradiation was stronger than those of control and irradiation-alone, similar to the results using cell lysate immunoprecipitated by antibody against EGFR (Figure 5C). This indicates that the resultant lysate immunoprecipitated by an antibody against HER2 included EGFR–cetuximab complexes even after cetuximab treatment with and without irradiation. Considering the results of Figure 5B and Figure 5C, cetuximab did not completely inhibit hetero-dimerization between EGFR and HER2.

To confirm the correlation between the inhibitory effect of cetuximab on radiation-induced homo- and/or hetero-dimerization and the inhibition of the autophosphorylation of EGFR and HER2, the changes in the phosphorylation of EGFR and HER2 caused by irradiation with and without cetuximab treatment were evaluated. As shown in Figure 5D, radiation induced the autophosphorylation of HER2, similarly to EGFR; cetuximab treatment in combination with radiation did not completely inhibit the radiation-induced autophosphorylation of HER2 consistent with incomplete inhibition of the hetero-dimerization of EGFR and HER2, but cetuximab treatment did inhibit the radiation-induced autophosphorylation of EGFR.

Discussion

It is well-recognized that EGFR and its related signal transduction pathways may serve as mediators of resistance

to cancer treatment, such as radiation therapy and chemotherapy. Regarding the link between the EGFR expression level and cellular resistance to radiation, we showed that the degree of radioresistance is positively-correlated with the magnitude of EGFR overexpression (7). This pre-clinical correlation was also confirmed by the clinical results of studies on head and neck cancer. Ang *et al*. analyzed the correlation between EGFR expression and radiotherapeutic outcomes and reported that the prognosis of patients with a high EGFR expression level after radiation therapy was poorer than that of patients with relatively weak EGFR expression levels (13). Based on these pre-clinical and clinical investigations, EGFR and its related signal transduction pathways are regarded as valuable targets for increasing radiosensitivity. An important aspect of the use of EGFR and its related signal transduction pathways as therapeutic targets is that ionizing radiation induces the activation of RTKs, including EGFR, and their downstream pathways, resulting in a pro-proliferative and anti-apoptotic responses, especially in tumors overexpressing EGFR. To target RTKs and their related signal transduction pathways, specific antibodies such as cetuximab trastuzumab or tyrosine kinase inhibitors such as ZD1839 and erlotinib have already been used. We reported that the tyrosine kinase inhibitor genistein, and the selective inhibition of EGFR, ERK or PI3K using specific kinase inhibitors (AG1478, PD98059 or LY294002, respectively) synergistically potentiated radiation-induced cell killing in human esophageal cancer cell lines (14, 15). Concerning the radiosensitizing effect of cetuximab, Huang *et al*. demonstrated that cetuximab enhanced the radiosensitivity of squamous cell carcinoma cell lines derived from human head and neck cancer by altering cell-cycle progression and inducing apoptosis (16). Bonner *et al*. also demonstrated that cancer cell lines transfected with EGFR expression vectors are more radioresistant than parental lines, and the augmentation of EGFR expression using an adenoviral vector approach resulted in a greater radiosensitization after anti-EGFR therapy compared with that in cells that were not treated with the adenoviral vector (17-19). This result indicates that the EGFR expression level or the activation status influences the radiosensitizing effect of cetuximab.

In the mechanism underlying the modification or alteration of the radiation response through EGFR and its signal transduction pathways, radiation-induced autophosphorylation or the activation of EGFR is the critical event. For wild-type EGFR, the dimerization of the receptor is a prerequisite for the activation of the intracellular kinase domain and EGFR dimerization precedes the autophosphorylation of the tyrosine residue. The dimerization of EGFR leads to the stimulation of the tyrosine kinase activity of the intracellular kinase domain, resulting in the phosphorylation of specific tyrosine residues on the C-terminal tail of the receptor. However, whether radiation induces the dimerization of EGFR in a manner similar to that induced by ligand binding has not been fully elucidated, although radiation-induced autophosphorylation has been reported. The results of this study demonstrated that

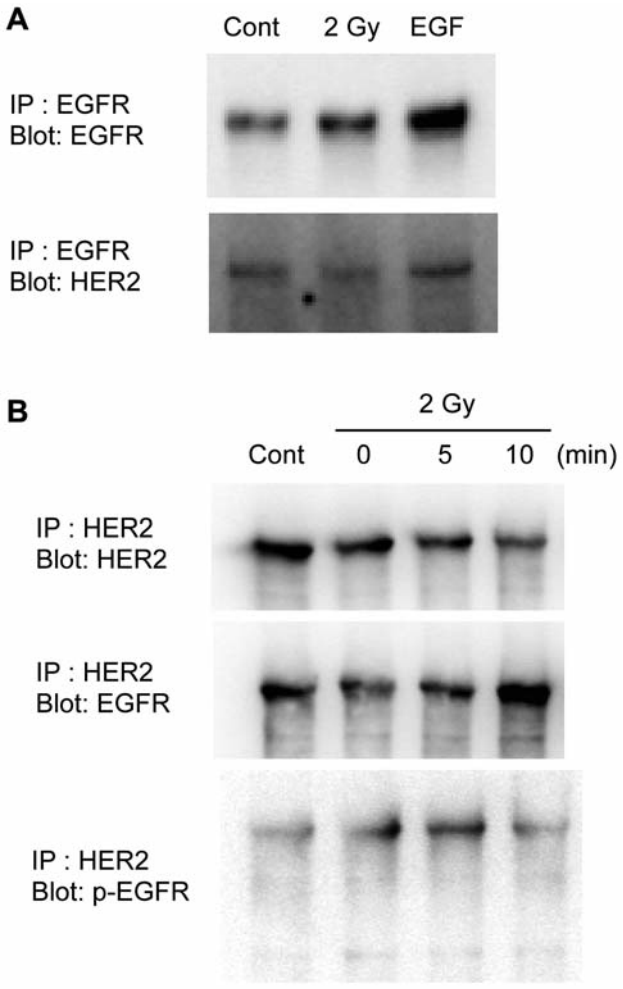


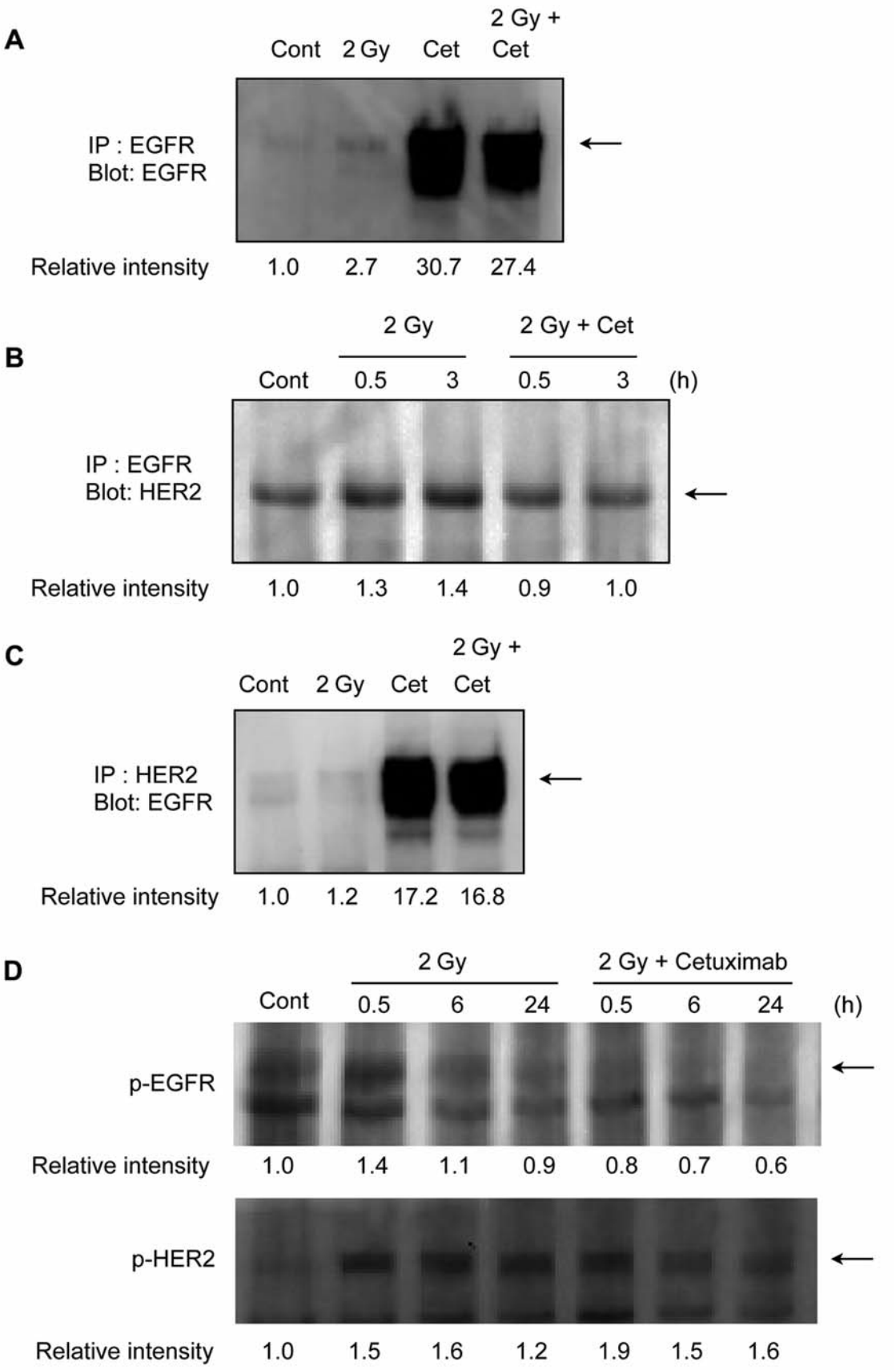
Figure 4. *Radiation-induced homo-dimerization of epidermal growth factor receptor (EGFR) and hetero-dimerization between EGFR and HER2/neu. A: Radiation-induced homo-dimerization of EGFR. Samples for the immunoprecipitation (IP) analysis were obtained soon after irradiation at a dose of 2 Gy or EGF stimulation at a concentration of 100 ng/ml for 3 min; the cell lysates were then immunoprecipitated using an antibody against EGFR. Equal amounts of the resultant proteins were subjected to western blot analysis as described in the Materials and Methods section. Irradiation at a dose of 2 Gy or EGF stimulation increased the band intensity, compared with no treatment, indicating that irradiation induced the homo-dimerization of EGFR similar to that induced by EGF stimulation. B: Radiation-induced hetero-dimerization between EGFR and HER2. Samples for the immunoprecipitation analysis were obtained at the indicated time points after irradiation at a dose of 2 Gy, and cell lysates were immunoprecipitated using antibody to HER2. Equal amounts of the resultant proteins were subjected to western blot analysis, as described in the Materials and Methods section, and the membranes were blotted with antibodies against EGFR, HER2 or phospho EGFR.*

the irradiation of A431 cells at a dose of 2 Gy induced the autophosphorylation of EGFR (phosphorylation of the intracellular kinase domain) and the activation of downstream signal transduction. Consistent with autophosphorylation, radiation also induced EGFR homo-dimerization. In addition to the homo-dimerization of EGFR, the results of the immunoprecipitation analysis demonstrated that irradiation at a dose of 2 Gy induced hetero-dimerization between EGFR and HER2. Liu *et al*. reported that the majority of EGFR and ERBB molecules have a homo- or hetero-dimeric structure under physiological conditions (20). Hence, it would be more appropriate to report that irradiation increased EGFR homo- and hetero-dimerization. Consistent with the inhibition of the autophosphorylation of EGFR, cetuximab also inhibited the radiation-induced homo-dimerization of EGFR. However, cetuximab inhibited radiation-induced hetero-dimerization between EGFR and HER2 incompletely, consistent with incomplete inhibition of radiation-induced autophosphorylation of HER2. Considering these results, the inhibition of radiation-induced homo- and/or hetero-dimerization between EGFR and/or HER2 by cetuximab might be involved in the mechanism underlying the strengthening of radiosensitivity, although more detailed investigation is needed to clarify the correlation between the inhibitory effect of cetuximab on the hetero-dimerization of EGFR and HER2 and the inhibition of radiation-induced HER2 autophosphorylation. To our knowledge, few studies have examined the correlation between radiation-induced homo- and/or hetero-dimerization of EGFR and the enhancement of radiosensitivity; hence, the results of the present study should provide with a valuable insight into the role of the receptor dimerization status in the cellular response to radiation and EGFR-targeting agents. Several investigations have suggested that targeting homo- or hetero-dimerization between EGFR and HER2 may be an effective treatment approach, demonstrating that the combined inhibition of EGFR and HER2 results in greater tumour growth inhibition or radiosensitivity than the blockade of either receptor alone (21-23).

Specific antibodies for EGFR, such as cetuximab, mediate their function through direct interaction with the EGF-binding site, blocking the ligand's ability to bind to EGFR (24). Hence, the sensitizing effect of specific antibodies for EGFR, such as cetuximab, may be greater in cancer cells that exhibit high levels of EGFR expression or radiation-induced autophosphorylation of the kinase domain than in cancer cells with a low EGFR expression level. This interpretation was confirmed in a study reported by Bonner *et al*., which demonstrated that the augmentation of EGFR expression using an adenoviral vector approach resulted in greater radiosensitization induced by an anti-EGFR antibody (cetuximab) than in cells that were not treated with the adenoviral vector (18, 19). This outcome was mainly attributed to the increase in EGFR expression, which augmented the responsiveness to the radiosensitizing properties of anti-EGFR treatments. Although Bonner *et al*. did not mention a conformational change in the extracellular domain of EGFR, the primary mechanism of cetuximab is the blockage of ligand-stimulated EGFR signaling through a direct interaction with the EGF-binding site (25, 26). Li *et al*. demonstrated that cetuximab interacts exclusively with domain III of EGFR, partially occluding the ligand binding region on this domain and sterically preventing the receptor from adopting the extended conformation required for dimerization (27). Hence, the inhibition or modification of the dimerization of EGFR is an important process in the antitumor effect of cetuximab.

The major limitation of this study is that we have not yet examined the radiation-induced conformation change in the extracellular domain of EGFR, although we have confirmed the formation of ligand-independent radiation-induced homo- and hetero-dimerization and the inhibitory effect of cetuximab. The changes in the conformation of the extracellular domain of EGFR are flexible and dynamic. The extracellular domain of EGFR consists of four sections, two ligand domains and two cysteine-rich domains, and it can adopt two distinct conformations: a tethered conformation and an untethered or extended conformation (28). In cells with EGFR overexpression, there is an increase in untethering as a result of ligand-independent EGFR activation and changes in glycosylation (29). In addition, Tao and Maruyama demonstrated that all four ERBB receptors have inactive, homo- and/or hetero-dimeric structures on the cell surface in the absence of bound ligands (30), and EGFR has been reported to exhibit homodimeric structures in the absence of bound ligand in a temperature-dependent manner: 13% at 4˚C, 36% at 20˚C and 69% at 37˚C (31, 32). In addition, a ligand-independent association of EGFR and HER2 has also been demonstrated (33). Therefore, investigating the exact conformational change induced by radiation with and without cetuximab treatment and the difference in the conformations between the ligand-unbound dimerization and the radiation-induced dimerization of EGFR may provide with valuable information regarding the biological implications of therapeutic targets. Such information could then be used to explore whether radiation-induced homo- and hetero-dimerization of the receptors represents an active conformation, compared with that in the steady state or during active dimerization induced by ligand binding.

In conclusion, the results of this investigation demonstrated that irradiation at a dose of 2 Gy induced the homo-dimerization of EGFR and hetero-dimerization between EGFR and HER2 compared with that in the steady state. Furthermore, the inhibitory effect of cetuximab on dimerization was well-correlated with the inhibition of the autophosphorylation of EGFR and HER2. These results suggest that the modification of radiation-induced homo- and hetero-dimerization between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EGFR and HER2 by cetuximab might be involved in the mechanism underlying the strengthening of radiosensitivity; hence, the modulation or inhibition of the receptor dimerization status may be an effective approach to enhancing the radiosensitivity of cancer cells.

Conflicts of Interest

None.

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Figure 5. *Effect of cetuximab (Cet) combined with radiation on hetero-dimerization between epidermal growth factor receptor (EGFR) and HER2/neu. A: Effect of cetuximab on homo-dimerization of EGFR and hetero-dimerization between EGFR and HER2. The samples for the immunoprecipitation (IP) analysis were obtained soon after irradiation at a dose of 2 Gy with and without cetuximab at a concentration of 10 μg/ml or the incubation of cells with cetuximab for 72 h; the cell lysates were then immunoprecipitated using antibody to EGFR. B: Changes in the formation of hetero-dimerization between EGFR and HER2 after irradiation at a dose of 2 Gy with and without cetuximab. Samples for the immunoprecipitation analysis were obtained at 0.5 and 3 h after irradiation at a dose of 2 Gy with and without cetuximab at a concentration of 10 μg/ml and then cell lysates were immunoprecipitated by an antibody against EGFR. C: Effect of cetuximab on homo-dimerization of EGFR and hetero-dimerization between EGFR and HER2. The samples for the immunoprecipitation analysis were obtained soon after irradiation at a dose of 2 Gy with and without cetuximab at a concentration of 10 μg/ml or the incubation of cells with cetuximab for 72 h; the cell lysates were then immunoprecipitated using antibody to HER2. D: Inhibitory effect of cetuximab on radiation-induced autophosphorylation of EGFR and HER2. Cells were irradiated at a dose of 2 Gy with and without cetuximab, and samples for western blotting were obtained at 0.5, 6 and 24 h after irradiation The cells were irradiated for 24 h after the start of incubation with cetuximab at a concentration of 10 μg/ml, and samples for western blotting were obtained at the time-points indicated after the irradiation.*

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