Radiosensitivity Enhancement by Combined Treatment of Celecoxib and Gefitinib on Human Lung Cancer Cells

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Abstract **Purpose:** To characterize the radiation-enhancing effects and underlying mechanisms of combined treatment with celecoxib, a cyclooxygenase-2 selective inhibitor, and gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, in human lung cancer cells.

Experimental Design: Clonogenic cytotoxicity assays and clonogenic radiation survival assays after treatments with celecoxib and gefitinib with or without radiation were done on three human lung cancer cell lines. Synergisms after combined treatment with celecoxib, gefitinib, and radiation were investigated using isobologram and statistical analyses according to an independent action model. Alterations in apoptosis and cell cycle were measured to identify the mechanisms underlying the cell killing or radiation-enhancing effects of celecoxib and gefitinib combination treatment. Western blots for phosphorylated EGFR, EGFR, cyclooxygenase-2, and G_2 checkpoint molecules were conducted after treatment with celecoxib and/or gefitinib with or without radiation.

Results: Combination celecoxib, gefitinib, and radiation treatments were shown to be synergistic in causing clonogenic cell deaths in all cell lines tested, but the nature of synergism was cell type specific. The combined drug treatments induced apoptosis in an additive manner in A549 cells and in a synergistic manner in NCI-H460 and VMRC-LCD cells. Celecoxib or gefitinib attenuated radiation-induced G_2-M arrest, and combined drug treatment additively attenuated radiation-induced G_2-M arrest in all cell lines. Radiation-induced checkpoint kinase (Chk) 1 and Chk2 phosphorylation were inhibited by celecoxib and gefitinib treatment, respectively.

Conclusions: Combined celecoxib and gefitinib treatments were shown to synergistically enhance the effect of radiation on lung cancer cells. The mechanisms underlying these synergistic effects seem to involve the synergistic enhancement of apoptosis and cooperative attenuation of radiation-induced G_2-M arrest, possibly via Chk1 and Chk2 inhibition, by the combined drug treatments.

Lung cancer is the most commonly diagnosed cancer and the leading cause of cancer-related death in the world (1). With the existing therapeutic efforts, patients with lung cancer have a 5-year survival rate of <15%, and this statistic has changed minimally in the last 25 years, underscoring the need for new therapeutic strategies. Understanding the molecular mechanisms involved in the pathogenesis and proliferation of lung cancer may provide opportunities to develop innovative therapies for this fatal disease (2, 3). Epidermal growth factor receptor (EGFR) and cyclooxygenase (COX)-2 are two of the recently discovered novel targets for lung cancer treatments (4).

Of several biological markers, EGFR has received considerable attention during the last decades. EGFR is a 170-kDa membrane-bound protein encoded by 28 exons on chromosome 7p12 and is a member of the tyrosine kinase (TK) family, which belongs to a subfamily of four closely related receptors (HER-1/ErbB1, HER-2/*neu*/ErbB2, HER-3/ErbB3, and HER-4/ErbB4). The receptors exist as inactive monomers. On binding to ligands, such as epidermal growth factor and transforming growth factor-α, the receptors undergo conformational changes that facilitate homodimerization or heterodimerization. Growth factor–induced receptor dimerization of EGFR is followed by intermolecular autophosphorylation of key tyrosine residues in the activation loop of the catalytic TK domain. A range of downstream intracellular signaling pathways are subsequently activated, which lead to increased DNA replication and the stimulation of cellular differentiation and proliferation (5). EGFR is an oncogene capable of inducing cancer when aberrant (6–8) and has been shown to play a key role in the development and progression of human epithelial cancers (9). Increased EGFR expression has been observed in many experimental cancer cell lines and human tumors, including lung cancers, and has been associated with resistance

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to cytotoxic drugs and radiation (9, 10). In addition, EGFR inhibitors have been shown to enhance the effects of radiation (10).

COX is a key enzyme that catalyzes the conversion of arachidonic acid to prostaglandins as well as other prostanoids. To date, two COX isoforms have been identified. COX-1 is constitutively expressed in a variety of cell types and seems to be intimately involved in the homeostasis of several physiologic functions, whereas COX-2 is an inducible enzyme, which is regulated by various factors, including cytokines, growth factors, and tumor promoters (11, 12). Increased COX-2 expression has been observed in a host of tumor types in humans and animals, including lung cancer. COX-2 selective inhibitors have been reported to prevent carcinogenesis and have also been shown to ameliorate the growth rate of tumor cells both *in vitro* and *in vivo*. In addition, COX-2 selective inhibitors are known to sensitize tumor cells to chemotherapeutic agents and ionizing radiation (13).

EGFR and COX-2 are intimately related within cells. Activation of EGFR signaling leads to increased mitogen-activated protein kinase activity resulting, in turn, in activator protein-1–mediated induction of *COX-2* transcription. Increased *COX-2* transcription results in enhanced production of prostaglandins, including prostaglandin E_2. In addition, there also is growing evidence that COX-2-derived prostaglandin E_2 can activate EGFR signaling and thereby stimulate cell proliferation. The mechanisms by which this occurs seem to be complex and context specific. Regardless of the precise mechanism, exposure to COX-2-derived prostaglandin E_2 may initiate a positive feedback loop whereby activation of EGFR results in enhanced expression of COX-2 and increased synthesis of prostaglandins. This, in turn, leads to further enhancement of EGFR activity. However, the interactions between EGFR and COX-2 in the cells still remain largely undefined (reviewed in ref. 14).

Because both EGFR inhibitors and COX-2 inhibitors have been shown to enhance the effects of radiation in cancer cells and EGFR and COX-2 are intimately related within cells, as discussed above, it may then be expected that the combined treatment of EGFR inhibitors and COX-2 inhibitors can radiosensitize cancer cells in a synergistic or, at least, additive manner. Therefore, we assessed the effects of combined treatment with celecoxib, a clinically available COX-2 selective inhibitor, and gefitinib, a clinically available EGFR-TK inhibitor, on the radiosensitivity of lung cancer cells to determine whether this drug combination may be beneficial for lung cancer patients undergoing radiotherapy.

Materials and Methods

Reagents. Gefitinib was kindly provided by AstraZeneca UK Ltd. (London, United Kingdom) and celecoxib was kindly provided by Pharmacia Corp. (Skokie, IL).

Cell culture. A549 human lung adenocarcinoma and NCI-H460 human lung large cell carcinoma cell lines were acquired from the American Type Culture Collection (Manassas, VA), and VMRC-LCD human lung adenocarcinoma cell line was acquired from the Japanese Cancer Research Resources Bank (Osaka, Japan). The cells were cultured in the recommended medium supplemented with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD), 50 units/mL penicillin (Life Technologies), and 50 μg/mL streptomycin (Life Technologies). Cells were carried for no more than eight passages, and only cultures that were <90% confluent were used in all of the experiments.

Immunoblotting. Immunoblotting was done as described previously (15). Sources of primary antibodies are as follows: rabbit polyclonal anti-COX-2 (Cayman Chemical, Ann Arbor, MI); rabbit polyclonal anti-EGFR (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse monoclonal anti-phosphorylated EGFR (Transduction Laboratories, Lexington, KY); and rabbit polyclonal anti-phosphorylated Ser^{345} checkpoint kinase (Chk) 1 (pChk1), anti-phosphorylated Thr^{68} Chk2 (pChk2), anti-phosphorylated Ser^{216} Cdc25C, anti-phosphorylated Tyr^{15} cyclin-dependent kinase 1 (Cdk1), anti-phosphorylated Thr^{161} Cdk1, mouse monoclonal anti-Cdk1, and anti–cyclin B1 (Cell Signaling Technology, Beverly, MA).

Clonogenic assay for cytotoxicity measurement and radiation survival experiment. Clonogenic assay was done as described previously (15). The surviving fractions in the cells exposed to radiation plus celecoxib (or gefitinib or celecoxib + gefitinib) were normalized by dividing by the surviving fraction obtained for celecoxib (or gefitinib or celecoxib + gefitinib) alone. We then calculated the dose enhancement ratio as the dose (Gy) for the radiation plus vehicle divided by the dose (Gy) for radiation plus celecoxib (or gefitinib or celecoxib + gefitinib; normalized for drug toxicity) at a surviving fraction of 0.1.

Detection of cell cycle changes and apoptosis via flow cytometry. In brief, 2.5×10^5 to 5×10^5 cells were plated into 25 cm^2 flasks for the determination of each data point. After 24 hours, the cells were exposed to the appropriate concentrations of celecoxib and/or gefitinib or vehicle (DMSO) for 4 hours and then exposed to graded doses of γ-rays. The cells were further incubated in medium that contained either the drug(s) or the vehicle for 20 to 68 hours. For cell cycle measurement, the cells were harvested and fixed with 75% ethanol at −20°C overnight and then incubated with 10 μg/mL propidium iodide (Sigma) and 5 μg/mL RNase A (Amresco, Solon, OH) at room temperature for 3 hours. The number of cells at each cell cycle was evaluated with the FACSCalibur system (Becton Dickinson, San Jose, CA). For apoptosis measurement, the cells were harvested and stained with Annexin V/propidium iodide for 15 minutes using Annexin V-Fluos Staining kit (Roche Applied Science, Penzberg, Germany) following the manufacturer's instructions. The number of cells that had undergone apoptosis was evaluated with the FACSCalibur system. Cells that were Annexin V positive and propidium iodide negative were labeled early apoptosis. Propidium iodide–positive cells were not included in the analysis. Error bars were also calculated as SE by the pooling of the results of three independent experiments.

Isobologram analysis. The combined effects of celecoxib and/or gefitinib and/or radiation on clonogenic cell survival were analyzed using an improved isobologram method as described previously (16–18). The "envelope of additivity" in an isobologram is an area enclosed by mode I and mode II isoeffect lines, which are calculated based on additive and independent action models, respectively. If the agents are acting additively by similar mechanisms (additive action model), combined data points will lie near the mode II line. In contrast, if the agents are acting additively by independent mechanisms (independent action model), combined data points will lie near the mode I line. If a data point lies left or right of the envelope of additivity, then the combined effect for that point can be considered "synergistic" or "antagonistic," respectively, regardless of the real action mechanisms of the combined agents. However, a data point that lies within the envelope of additivity needs careful discussion to determine whether the point represents "mechanistically" additive action. The envelope of additivity is an extensively calculated area to rule out all possible additive actions of the combined agents and should not be considered as a reliable definition of additivity (19, 20). Therefore, action mechanisms of the agents in combination have to be considered in analyzing the data points within the envelope of additivity. It has been experimentally verified that the combination of dissimilarly acting agents is predictable by the independent action model (21–23). Because the three agents used in our experiments have their own distinct known targets (COX-2 for celecoxib, EGFR-TK for gefitinib, and DNA damage for radiation), we assumed that celecoxib, gefitinib, and

radiation in combination will act by independent mechanisms if they do not interact. Therefore, the mode I line in the isobologram was considered as the expected isoeffect line for our case when the agents are acting additively. Accordingly, when a data point was near to or on a mode I line, it was considered "additive," but when a point was to the left of the mode I line it was considered "synergistic" even if the point was within the envelope of additivity.

For three-agent combination, expected clonogenic survival values for combined treatments of the three agents were calculated according to the independent action model, for the same reason discussed above, as described in previous publications. This calculation method is same as the one for calculation of a mode I line or surface in two- or three-dimensional isobolograms. In brief, an expected clonogenic cell survival value after treatment with combined agents at certain concentrations or doses of each agent can be calculated simply by multiplication of surviving fractions (which are equal to 1-effect, the effect being clonogenic cell death in our experiments) observed after treatment with each agent alone at the same concentrations or doses used in the combined treatments (22, 23). Calculated (expected) values were compared with the respective observed values after combined treatments with three agents using independent t test. When $P \leq 0.05$, the observed combined effects were considered significantly different from expected values and defined as synergistic when the observed surviving fraction is less than the expected surviving fraction or antagonistic when the observed surviving fraction is greater than the expected surviving fraction. When $P > 0.05$, the observed values were considered additive. This statistical comparison of expected and observed values was also done for two-agent combinations when needed.

Statistical analysis. Synergism on apoptosis or cell cycle arrest in the combined treatments was tested for as described previously (24) after normalization of the data by subtracting control values. $P \leq 0.05$ was considered statistically significant and therefore synergistic or antagonistic, and $P > 0.05$ was considered additive.

Results

EGFR and COX-2 expression levels in each cancer cell line. We determined EGFR and COX-2 expression levels in each cell line as follows after Western blot analyses: A549 cells, EGFR+/COX-2+; NCI-H460 cells, EGFR+/COX-2−; VMRC-LCD cells, EGFR−/COX-2− (Fig. 1).

Cytotoxicity of celecoxib and/or gefitinib on lung cancer cells. To assess the cytotoxicities of celecoxib or gefitinib on each cancer cell line, the cells were exposed to various celecoxib or gefitinib dosages for 72 hours and then permitted to form colonies in drug-free medium. The average 25% (IC_{25}) and 50% (IC_{50}) inhibitory concentrations for clonogenic cell death in three tested cell lines were 40 and 50 μmol/L for celecoxib and 10 and 15 μmol/L for gefitinib, respectively. To assess the combined cytotoxicity of celecoxib and gefitinib, each cell line was exposed to 40 μmol/L celecoxib and 10 μmol/L gefitinib (designated DC_{25}; drug combination at the IC_{25} concentrations of each drug) or 50 μmol/L celecoxib and 15 μmol/L gefitinib (designated DC_{50}; drug combination at the IC_{50} concentrations of each drug) for 72 hours and then permitted to form colonies in drug-free medium. To evaluate the synergism of celecoxib and gefitinib treatment on clonogenic cell death, isobologram analyses were done using the dose-response curves of each agent (data not shown) at surviving fractions shown after DC_{25} or DC_{50} treatments in each cell line. All data points, except for DC_{25} treatment in VMRC-LCD cells, were shown to be within the envelope of additivity. Combined cytotoxicities after DC_{25} or DC_{50} treatments in A549 cells were shown to be close to the mode I line and were shown to be additive by statistical comparison of expected and observed values according to the independent action model (Fig. 2; Supplementary Fig. S1, top). In contrast, data points after DC_{25} or DC_{50} treatments in NCI-H460 cells fell to the left of the mode I line and were shown to be synergistic by statistical comparison of expected and observed values (Fig. 2; Supplementary Fig. S1, middle). DC_{25} treatments showed antagonistic action by isobologram analysis of VMRC-LCD cells, but DC_{50} treatments were shown to be synergistic in these cells (Fig. 2; Supplementary Fig. S1, bottom).

Effects of celecoxib and/or gefitinib on the radiosensitivity of lung cancer cells. To assess and characterize the radiation-enhancing effects of celecoxib and/or gefitinib, the cells were exposed to graded doses of γ-radiation with average IC_{25} or IC_{50} concentrations of celecoxib or gefitinib or with DC_{25} or DC_{50} treatments for 72 hours. Radiation was administered 4 hours after the start of drug treatment. The cells were then permitted to form colonies in drug-free medium. Isobologram analyses were done at surviving fractions shown after treatment with radiation and average IC_{25} concentrations of celecoxib or gefitinib. To generate the data points for

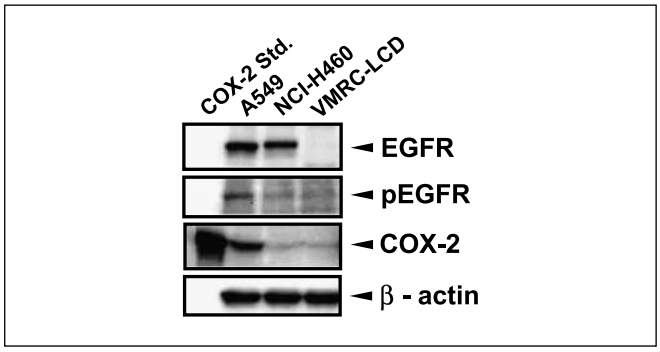

Fig. 1. Western blot analysis of EGFR, phosphorylated EGFR (*pEGFR*), or COX-2 expression in lung cancer cells. COX-2 Std., positive control of COX-2 (ovine COX-2 electrophoresis standard). A549 cells were determined as EGFR+/COX-2+, NCI-H460 as EGFR+/COX-2−, and VMRC-LCD as EGFR−/COX-2−. NCI-H460 and VMRC-LCD cells expressed minor amounts of COX-2.

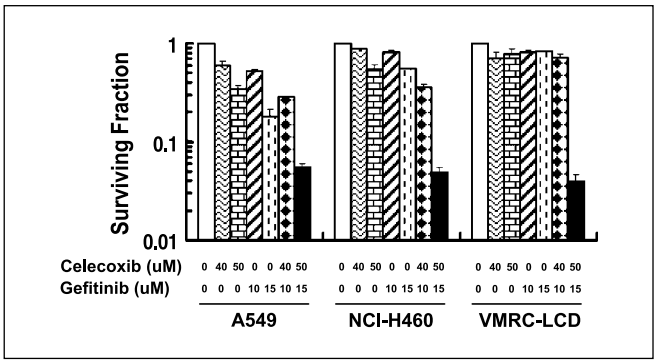

Fig. 2. Clonogenic cytotoxicity assays for celecoxib and/or gefitinib in lung cancer cells. Cells attached to the flasks were exposed to vehicle (DMSO) or average IC_{25} or IC_{50} concentrations of celecoxib or gefitinib for 72 hours and were then permitted to form colonies in drug-free medium. Bars, SE of three independent experiments done in triplicate. Combined drug treatments were shown to cause additive clonogenic cell death in A549 cells, whereas the same treatments were shown to cause synergistic clonogenic cell death in NCI-H460 and VMRC-LCD cells.

radiation and IC_{50} concentrations of celecoxib or gefitinib in the above isobolograms, radiation doses needed to cause the respective surviving fractions in combination with drugs were extrapolated from the dose-response curves drawn after graded doses of radiation and IC_{50} concentrations of either drug treatment.

After extensive isobologram analyses for combination celecoxib and gefitinib or radiation combined with either drug (Fig. 3; Supplementary Fig. S1), we concluded that the combined effects of these three agents seemed to follow independent action model when they are noninteractive, because the data points after two-agent combination treatments were frequently near to or on the mode I line. This finding is concordant with the hypothesis for the action mechanisms of the combined agents that we described in Materials and Methods. Therefore, we calculated expected clonogenic cell survival values after three-agent combined treatments according to independent action model and statistically compared these values with respective observed ones as described in Materials and Methods.

Celecoxib treatments at average IC_{50} concentrations were determined by isobologram and radiation survival analyses to synergistically augment the effects of radiation in all cell lines (Figs. 3, *top*, 4, and 5). However, celecoxib treatments at average IC_{25} concentrations were shown to synergistically augment the effects of radiation only in A549 cells and were shown to be additive in the other cells (Fig. 3, *top*). Gefitinib treatments at average IC_{25} or IC_{50} concentrations were not shown to augment the effects of radiation and were shown to be additive when combined with radiation in A549 cells (Figs. 3, *bottom left*, 4, and 5A). In contrast, gefitinib treatments at average IC_{50} concentrations were shown to synergistically augment the effects of radiation in NCI-H460 and VMRC-LCD cells. The combined effects with radiation and gefitinib treatment at average IC_{25} concentrations were additive or antagonistic, respectively, according to isobologram analyses in these cell lines (Figs. 3, *bottom middle* and *bottom right*, 4, and 5B and C).

Three-agent combined treatments (radiation with celecoxib and gefitinib) were shown to be synergistic in all cell lines when compared with expected additive values calculated according to independent action model, except for VMRC-LCD cells with radiation plus DC_{25} treatments (Table 1; Fig. 5). After extensive quantitative analyses using observed and expected values for two- or three-agent combined treatments, we concluded that the synergistic effects observed after three-agent combination treatment seemed to be the combined sums of synergistic two-agent interactions. First, because celecoxib-gefitinib and radiation-gefitinib combinations had additive effects in A549 cells (Figs. 3 and 5; Supplementary Fig. S1), the shown synergisms with three-agent combinations in these cells were thought to be

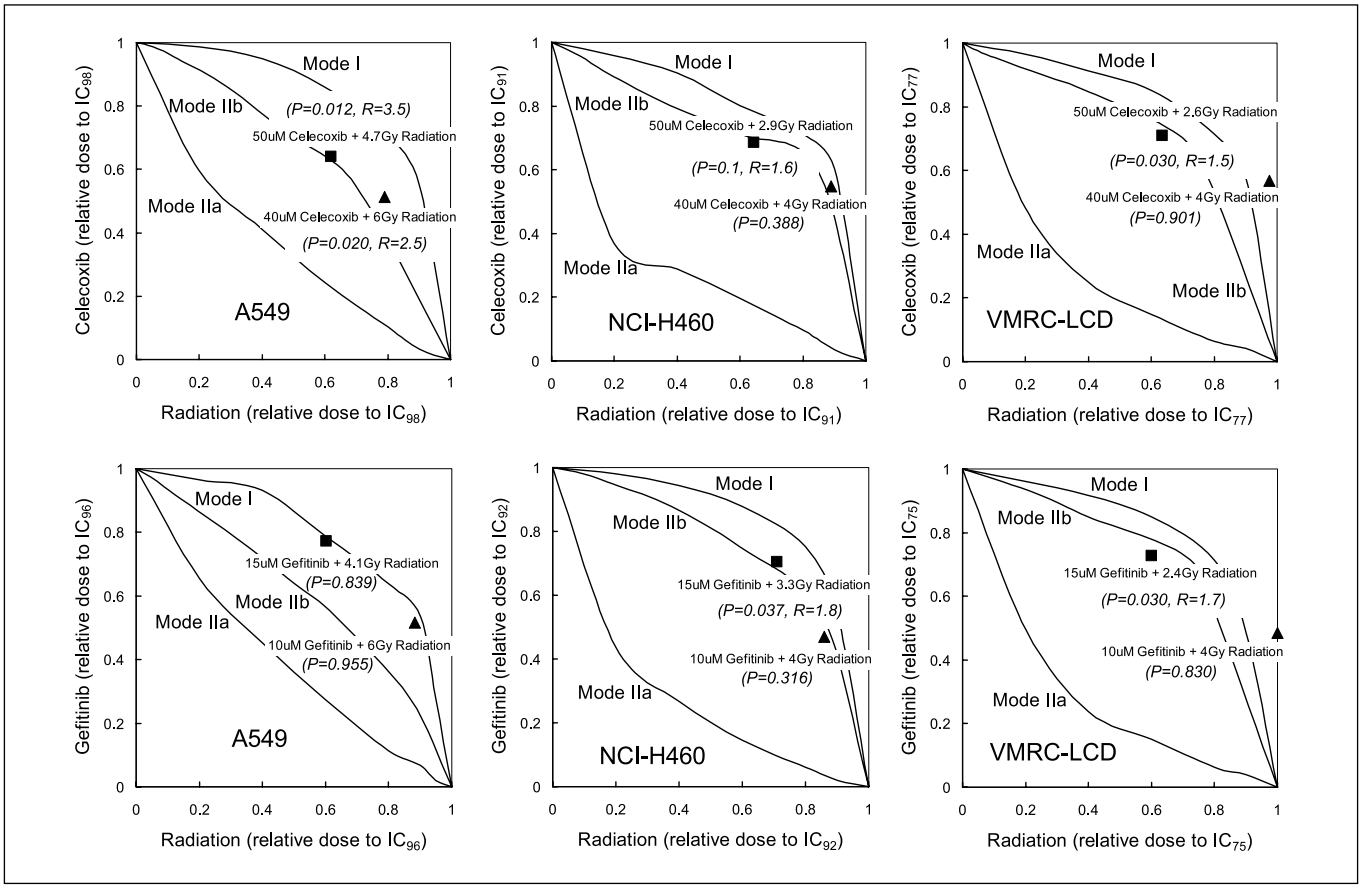

Fig. 3. Isobologram analysis for celecoxib, gefitinib, and radiation combination in lung cancer cells. Each isobologram was drawn at a surviving fraction that was observed after treatment with average IC_{25} concentrations of either drug + radiation. Mode I line was considered as the expected isoeffect line (when additive) according to independent action model. *P*s in parentheses were calculated from statistical comparison of expected and observed surviving fractions according to independent action model as described in Materials and Methods. *R*s in parentheses represent ratio of expected surviving fraction to observed surviving fraction.

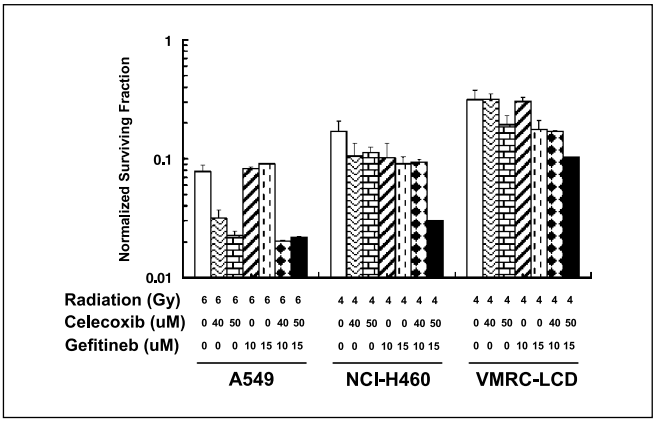

Fig. 4. Clonogenic radiation survival assays with celecoxib and/or gefitinib treatments in lung cancer cells. Cells attached to the flasks were treated with celecoxib and/or gefitinib for 4 hours, irradiated, rinsed after a 68-hour incubation in drug-containing medium, and allowed to form colonies in drug-free medium. Surviving fractions for radiation + celecoxib were normalized by dividing by the surviving fraction for celecoxib (or gefitinib or celecoxib + gefitinib) only. Bars, SE of three independent experiments done in triplicate. Synergistic radiation-enhancing effects have been shown in NCI-H460 and VMRC-LCD cells after celecoxib and gefitinib treatments.

attributable to a synergism between radiation and celecoxib. A three-agent combination (6 Gy radiation and DC_{50} treatment) enhanced the total effect by 4.5 times compared with the expected value (Table 1). The celecoxib-radiation combination at the same concentration and dose enhanced the effect by 3.5 times compared with the expected value (calculated data not shown), which is similar to the observed value after the three-agent combination treatment. Second, in NCI-H460 cells, a three-agent combination (4 Gy radiation and DC_{50} treatment) enhanced the total effect by 18.8 times compared with the expected value (Table 1). Celecoxib-gefitinib, celecoxib-radiation, and gefitinib-radiation combinations at the same concentrations and doses enhanced the effects by 6.5 times (Supplementary Fig. S1), 1.6 times, and 1.9 times (calculated data not shown), respectively, compared with the expected values. Therefore, theoretical combined synergistic values for the three-agent combination can be 19.8 times ($6.5 \times 1.6 \times 1.9$) higher than the expected value, which is similar to the value we observed. Third, in VMRC-LCD cells, a three-agent combination (4 Gy radiation and DC_{50} treatment) enhanced the total effect by 44.7 times compared with the expected value (Table 1). Celecoxib-gefitinib, celecoxib-radiation, and gefitinib-radiation combinations at the same concentrations and doses enhanced the effects by 18 times (Supplementary Fig. S1), 1.6 times, and 1.7 times (calculated data not shown), respectively. Therefore, theoretical combined synergistic values for the three-agent combination can be 49 times ($18 \times 1.6 \times 1.7$) the expected value, which is similar to the observed value. These findings may imply that the synergism between each two agents in our experiments is acting independent of one another and can be also combined in three-agent combinations by the independent action model.

Dose enhancement ratios after each drug treatment at IC_{50} concentrations or after DC_{50} treatments in the tested cell lines were determined and are presented in Table 2. They show that combined drug treatments can further enhance the effect of radiation compared with the enhancement of radiation effect due to each drug alone.

Effects of celecoxib and/or gefitinib with or without radiation on apoptosis induction in lung cancer cells. We then attempted to determine whether the additive or synergistic clonogenic cytotoxicities or radiation-enhancing effects of combined celecoxib and gefitinib treatments were attributable to additive or synergistic apoptosis induction in the tested cell lines. In brief, the cells were exposed to average IC_{50} concentrations of celecoxib (50 μmol/L) and/or gefitinib (15 μmol/L) or vehicle for 4 hours, irradiated with 0, 9, or 12 Gy γ-radiation, and further incubated in medium containing drug(s) or vehicle for 68 hours. The apoptotic cell fraction was then measured using flow cytometric analysis. Apoptotic values were normalized by subtracting their control values. Then, the normalized apoptotic values after combined treatments were compared with the sums of the normalized values after treatment with each agent alone.

DC_{50} treatment without radiation was shown to induce apoptosis in an additive manner in A549 cells when compared with each drug treatment separately (Fig. 6A). In contrast, the combined drug treatment was shown to induce apoptosis in a synergistic manner in NCI-H460 and VMRC-LCD cells (Fig. 6B and C). It is noteworthy that DC_{50} treatment increased apoptosis synergistically in VMRC-LCD cells, although separate treatments of each drug manifested negligible amounts of apoptosis compared with control cells (Fig. 6C).

Celecoxib treatment was shown to synergistically increase radiation-induced apoptosis in all cell lines tested when compared with celecoxib or radiation separately (i.e., in Fig. 6, the portion of the column above the dashed line for celecoxib + radiation treatment is longer than the portion of the column above the dotted line for celecoxib alone treatment). In contrast, gefitinib treatment was shown to synergistically increase radiation-induced apoptosis in A549 cells but reduce radiation-induced apoptosis in other cell lines (Fig. 6).

DC_{50} treatment with radiation was shown to induce apoptosis in less than additive or additive manners in A549 cells (Fig. 6A) when compared with each drug alone with radiation. The same treatment was shown to induce apoptosis in a synergistic manner in NCI-H460 and VMRC-LCD cells but not in NCI-H460 cells subjected to 12 Gy radiation (Fig. 6B and C).

Effects of celecoxib and/or gefitinib with or without radiation on cell cycle regulation in lung cancer cells. To determine whether the clonogenic cytotoxicities or radiation-enhancing effects of celecoxib and gefitinib combination treatment were related to cell cycle regulation, cells were exposed to 50 μmol/L celecoxib and/or 15 μmol/L gefitinib or vehicle for 4 hours, irradiated with 0, 9, or 12 Gy γ-radiation, and then further incubated in medium containing drug(s) or vehicle for 20 hours, and the number of cells in each cell cycle phase was determined using flow cytometric analysis.

Celecoxib treatment alone was shown to induce G_0-G_1-phase delays in all cell lines tested (data not shown), and a synchronous G_2-M-phase delay was observed in VMRC-LCD cells (Fig. 7C). Celecoxib treatment was shown to reduce radiation-induced G_2-M prolongation in NCI-H460 and VMRC-LCD cells (Fig. 7B and C). Celecoxib treatment reduced the radiation-induced G_2-M prolongation in A549 cells after administration of 12 Gy radiation (Fig. 7A). Gefitinib treatment alone was not shown to alter cell cycle phases in A549 or NCI-H460 cells and was shown to induce prolongation of G_0-G_1

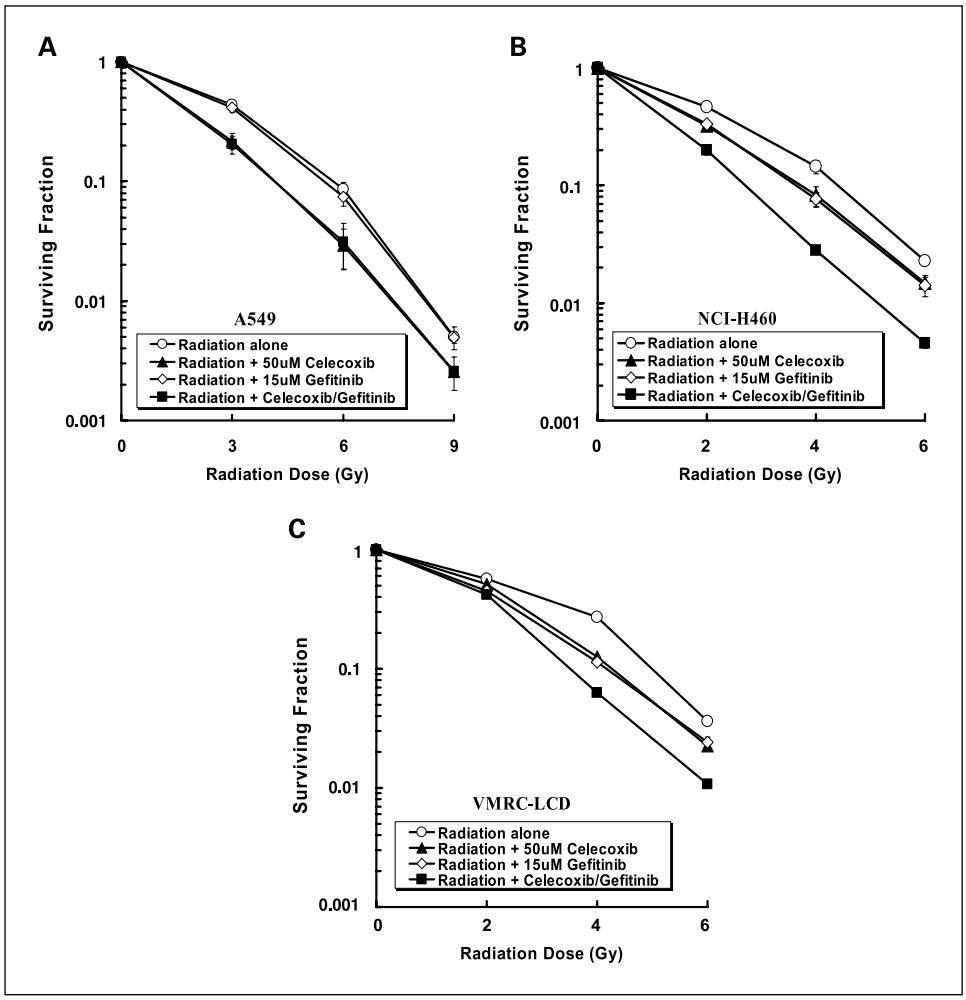

Fig. 5. Clonogenic survival curves for radiation + celecoxib and/or gefitinib treatments in A549 (*A*), NCI-H460 (*B*), or VMRD-LCD (*C*) cells. Cells attached to the flasks were treated with 50 μmol/L celecoxib and/or 15 μmol/L gefitinib for 4 hours, irradiated with graded doses of γ-rays, rinsed after a 68-hour incubation in drug-containing medium, and allowed to form colonies in drug-free medium. Surviving fractions for radiation + celecoxib were normalized by dividing by the surviving fraction for celecoxib (or gefitinib or celecoxib + gefitinib) only. Bars, SE of three independent experiments done in triplicate. The radiation-enhancing effect of celecoxib and gefitinib was comparable with that of celecoxib alone in A549 cells, but this drug combination enhanced the effect of radiation in a synergistic manner in NCI-H460 and VMRC-LCD cells.

phase in VMRC-LCD cells (data not shown). Gefitinib treatment was shown to mildly enhance radiation-induced G_2-M prolongation in A549 cells (Fig. 7A) but reduced the radiation-induced G_2-M prolongation in NCI-H460 and VMRC-LCD cells (Fig. 7B and C). DC_{50} treatment reduced the radiation-induced G_2-M prolongation in all cell lines in an additive manner when compared with each drug alone with radiation (Fig. 7A-C).

Because celecoxib and/or gefitinib treatments were shown to modulate radiation-induced G_2-M prolongation, we investigated

Table 1. Synergism analysis for three-agent combination by comparing expected and observed clonogenic surviving fractions after treatment with celecoxib and gefitinib with radiation according to independent action model in lung cancer cells

Treatment group	Expected surviving fraction*	Observed surviving fraction	Ratio expected/ observed surviving fraction	*P* value of difference†	Synergism
A549 cells					
DC_{25} + 6 Gy radiation	0.026 ± 0.005	0.006 ± 0.000	4.7	0.01	Synergistic interaction
DC_{50} + 6 Gy radiation	0.009 ± 0.003	0.002 ± 0.001	4.5	0.05	Synergistic interaction
NCI-H460 cells					
DC_{25} + 4 Gy radiation	0.114 ± 0.019	0.033 ± 0.004	3.5	0.05	Synergistic interaction
DC_{50} + 4 Gy radiation	0.056 ± 0.007	0.003 ± 0.001	18.8	0.005	Synergistic interaction
VMRC-LCD cells					
DC_{25} + 4 Gy radiation	0.181 ± 0.031	0.122 ± 0.014	1.5	0.276	Additive action
DC_{50} + 4 Gy radiation	0.201 ± 0.016	0.005 ± 0.001	44.7	0.007	Synergistic interaction

*Calculated according to independent action model as described in Materials and Methods.
†*P* value after statistical comparison of expected and observed value as described in Materials and Methods.

Table 2. Dose enhancement ratio for celecoxib, gefitinib, or combined drug treatment at surviving fraction = 0.1

Cell line	Celecoxib	Gefitinib	Combined
A549	1.39	1.06	1.39
NCI-H460	1.19	1.21	1.63
VMRC-LCD	1.16	1.20	1.43

the molecular pathways of G_2 checkpoint after treatments with these drugs and radiation. Cells were exposed to 50 μmol/L celecoxib and/or 15 μmol/L gefitinib or to the vehicle for 4 hours, irradiated with 9 Gy γ-radiation, and then further incubated in medium containing drug(s) or the vehicle. Total proteins were harvested at 1, 3, 6, 12, 15, 20, 24, 48, and 72 hours after radiation, and Western blot analyses for G_2 checkpoint molecules were done. pChk1 was increased at ~20 to 24 hours after radiation in all cell lines. Celecoxib treatment was shown to decrease pChk1 levels in all cell lines, and this finding was more evident when treatment was combined with radiation compared with radiation treatment alone. This decrease of pChk1 by celecoxib treatment was most evident at ~20 to 24 hours after radiation in all cell lines. In A549 cells, an upward band shift of pChk1 was noted after celecoxib treatment with or without radiation, indicating protein modification by this agent (Fig. 8). Increase of pChk2 was evident from 1 hour after radiation in all cell lines (data not shown). Gefitinib treatment was shown to decrease pChk2 levels in all cell lines when combined with radiation compared with radiation alone. This decrease of pChk2 by gefitinib started between 1 and 6 hours after radiation and peaked at ~20 to 72 hours according to cell line (representative blots at 20 hours after radiation in Fig. 8). No additive or synergistic decrease of pChk1 or pChk2 was noted after the combined celecoxib-gefitinib treatment. Changes in downstream molecules were complicated and cell type specific. Anti-phosphorylated Ser^{216} Cdc25C was decreased after celecoxib treatment, with or without radiation, in only A549 cells. In contrast, anti-phosphorylated Ser^{216} Cdc25C was decreased after gefitinib treatment, with or without radiation, in all cell lines. Total Cdk1 and cyclin B1 levels were decreased after celecoxib and radiation treatment in only A549 cells but were not changed in NCI-H460 and VMRC-LCD cells when compared with radiation alone. The total Cdk1 level was decreased after gefitinib and radiation treatment in A549 and NCI-H460 cells. The cyclin B1 level was not changed in any cell line after gefitinib treatment (Fig. 8).

Discussion

There have been few reports on the combined effects of COX-2 inhibitors and EGFR-TK inhibitors. Chen et al. and

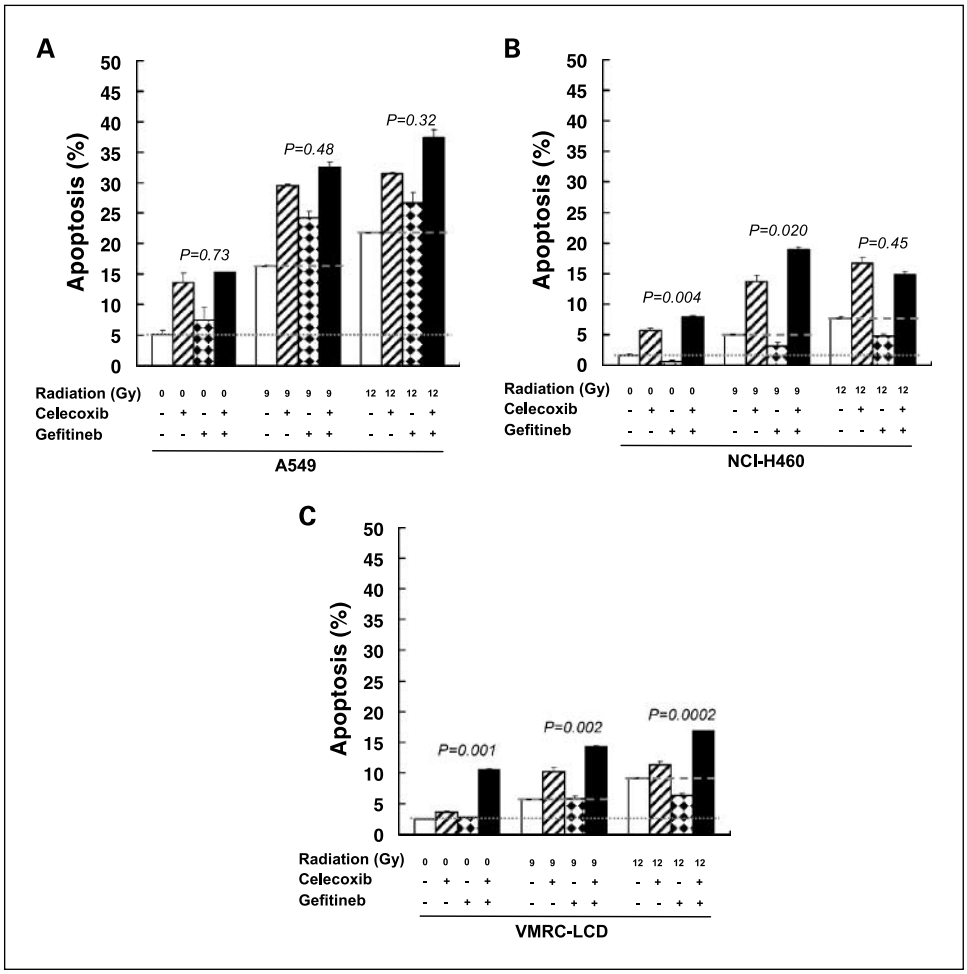

Fig. 6. Apoptosis induction after celecoxib and/or gefitinib treatments with or without radiation in A549 (*A*), NCI-H460 (*B*), or VMRC-LCD (*C*) cells. Attached cells were treated with 50 μmol/L celecoxib and/or 15 μmol/L gefitinib for 4 hours and then irradiated. The cells were harvested after an additional 68-hour incubation in medium containing the indicated drug(s). The fraction of apoptotic cells was measured using flow cytometry. Bars, SE of three independent experiments. Apoptotic values were normalized by subtracting control values (normalized values are equivalent to the portions of the column above the dotted line); the normalized apoptotic values were used for statistical analyses. Dashed lines are given to facilitate understanding of synergy analysis. The portion of each column below the dashed line is equivalent to apoptotic amount by radiation alone, and the portion above the dashed line is equivalent to apoptosis by drug(s) alone + by possible synergism. Normalized apoptotic values after combined treatments were compared with the sums of the normalized apoptotic values after treatment with each agent alone. In addition, the portion above the dashed line after three-agent combination treatments was compared with the sum of the same portions after treatment with each drug + radiation to investigate whether enhancement of apoptosis induced by radiation + two drugs is bigger than the sum of the apoptosis enhancement by radiation + each drug. If $P \leq 0.05$, significant synergy in drug combination on apoptosis induction or enhancement of radiation-induced apoptosis. Combined drug treatments were shown to enhance radiation-induced apoptosis in a less than additive or additive manner in A549 cells but in a synergistic manner in NCI-H460 and VMRC-LCD cells when compared with each drug alone with radiation.

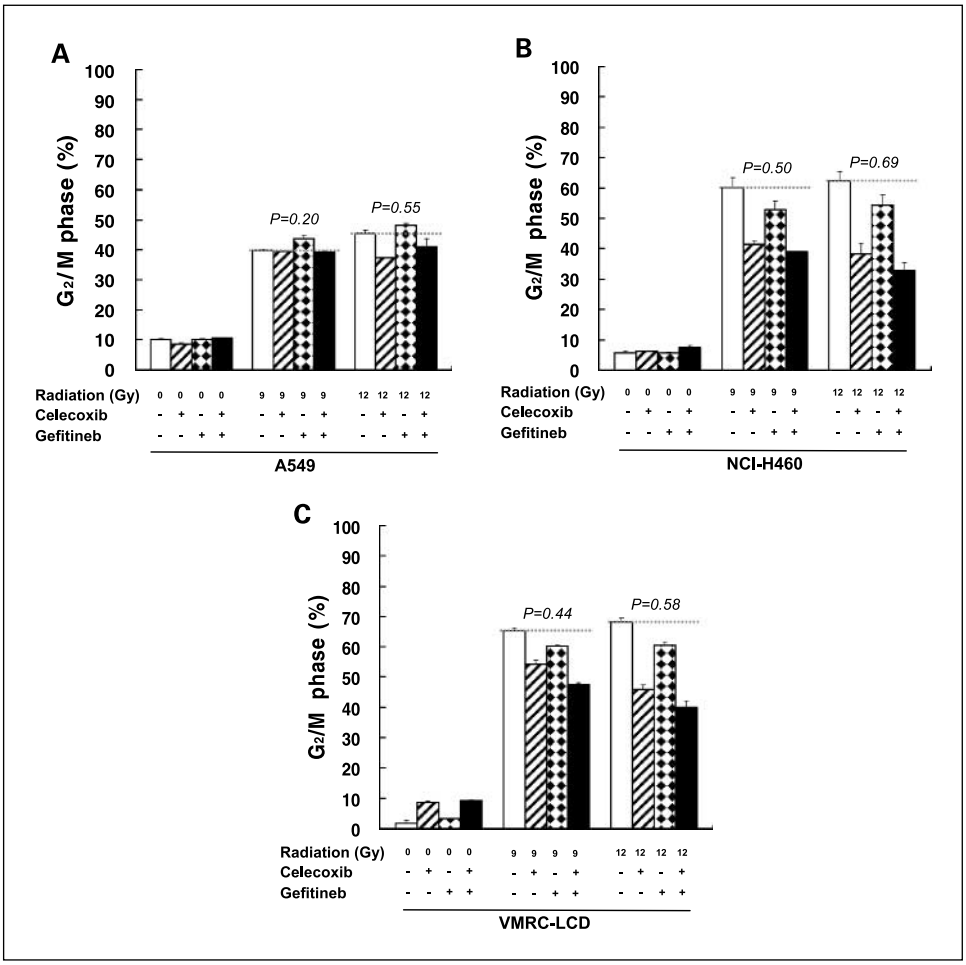

Fig. 7. Cell cycle changes after celecoxib and/or gefitinib treatments with or without radiation in A549 (*A*), NCI-H460 (*B*), or VMRC-LCD (*C*) cells. Attached cells were treated with 50 μmol/L celecoxib and/or 15 μmol/L gefitinib for 4 hours and then irradiated. The cells were harvested after an additional 20-hour incubation in medium containing the indicated drug(s), and the number of cells at each cell cycle phase was measured by flow cytometry. Bars, SE of three independent experiments. DC_{50} treatment reduced the radiation-induced G_2-M prolongation in all cell lines in an additive manner when compared with each drug alone with radiation.

Tortora et al. have described additive or synergistic antineoplastic effects after combined treatments containing EGFR-TK inhibitors and COX-2 inhibitors in head and neck cancer cell lines and in colon and breast cancer cell lines, respectively (25, 26). Because we also showed additive or synergistic anticancer effects with celecoxib and gefitinib treatments in lung cancer cell lines, it can be inferred that cooperative interactions between COX-2 inhibitors and EGFR-TK inhibitors may be a general phenomenon found in most cancer cell types.

Because COX-2 and EGFR are related within cells and combined treatments with inhibitors of these molecules have been shown to induce cooperative antineoplastic effects, as discussed above, and COX-2 inhibitors and EGFR-TK inhibitors have been shown to enhance the effects of radiation (10, 13), it may be expected that the combined treatment of COX-2 inhibitors and EGFR inhibitors can radiosensitize cancer cells in a cooperative manner. However, the effects of the combined treatment of COX-2 inhibitors and EGFR-TK inhibitors on radiosensitivity had not been addressed in previous studies. Therefore, we assessed the effects of combined treatment with celecoxib and gefitinib on the radiosensitivity of lung cancer cells. To determine whether the observed combined effects of celecoxib, gefitinib, and radiation are synergistic, additive, or antagonistic, it is essential to estimate additive values after these agents combine but do not interact. However, the estimation of additive values is a complicated subject because acting mechanisms of each agent in combination have to be considered to expect and calculate additivity, but these mechanisms are frequently poorly understood. There have been two major theoretical models proposed to understand and quantify additive action of combined agents. One is Loewe additivity and the other is Bliss-independent action. Numerous analytic methods, including isobologram, have been developed based on these two action models (detailed reviews in refs. 19, 27). Therefore, determining whether the effects of combined agents follow an additivity model or independent action model is critical to expect and calculate additive values. We decided to use an independent action model to calculate the additive combined effects of celecoxib, gefitinib, and radiation as described in detail in Materials and Methods. According to our results using isobologram and statistical analyses for expected and observed value comparison, our assumption for using independent action model seems to be valid for clonogenic cell death after treatments with these agents.

Statistical comparison of expected and observed values showed that combined treatment of celecoxib, gefitinib, and radiation caused clonogenic cell deaths in a synergistic manner in all cell lines. In addition, the degree of effect enhancement by synergistic interactions with three-agent combinations seemed to be the combined sum of synergistic two-agent interactions according to the calculated results shown in the current study. This may imply that each interaction between

two agents is acting independently in the three-agent combination and can be combined also by the independent action model to reveal synergisms in three-agent combinations. However, the nature of the synergy was different in A549 cells from that in NCI-H460 and VMRC-LCD cells. In A549 cells, gefitinib treatment alone did not radiosensitize the A549 cells in spite of significant cytotoxicity of this agent in these cells, nor did it manifest synergistic drug interactions with celecoxib in this cancer cell line. Therefore, the degree of radiation enhancement from this drug combination was comparable with that of celecoxib alone in A549 cells. This may indicate that COX-2-overexpressing cell lines, such as A549, may be able to be radiosensitized primarily by COX-2 inhibitors, and adding EGFR-targeted agents may not be beneficial for radiosensitization. By way of contrast, all two-agent combinations (i.e., celecoxib-gefitinib, celecoxib-radiation, and gefitinib-radiation combinations) exhibited synergistic interactions in NCI-H460 and VMRC-LCD cells, and combined treatments with three agents showed markedly enhanced (up to 18.8 and 44.7 times, respectively) cell-killing effects compared with expected values. These findings show that significant and synergistic interactions between celecoxib, gefitinib, and radiation can occur in some cancer cells. However, it is puzzling that an additive drug interaction between celecoxib and gefitinib was shown in COX-2- and EGFR-overexpressing A549 cells, whereas a synergistic interaction was shown in COX-2 and EGFR low-expressing VMRC-LCD cells. This perplexing issue will need to be solved with further experimentation. In summary, we have shown that the combined treatment of celecoxib and gefitinib can cooperatively enhance the effect of radiation in lung cancer cells, but the nature of cooperation seems to be specific to cell type. Because both drugs are orally active and have been shown to be relatively nontoxic in humans, this drug combination may be a good radiosensitizing agent for lung cancer patients and even for the case of additive radiosensitizations.

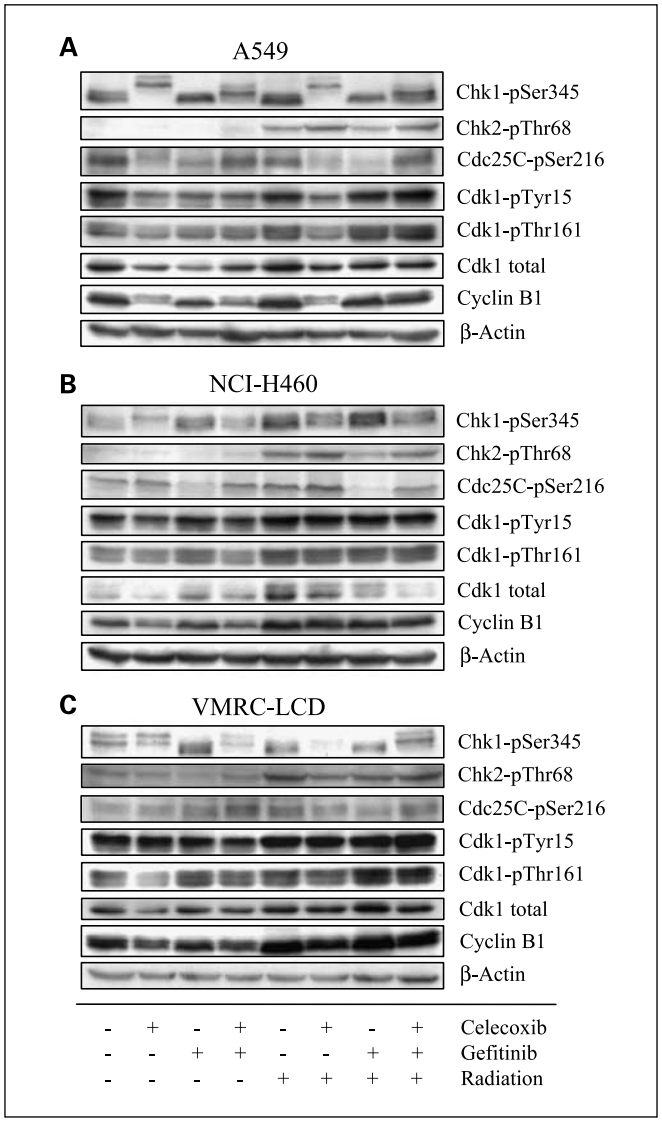

Fig. 8. Western blot analyses for G_2 checkpoint pathway evaluation after treatments with 50 μmol/L celecoxib and/or 15 μmol/L gefitinib with or without 9 Gy radiation in A549 (*A*), NCI-H460 (*B*), or VMRC-LCD (*C*) cells. Celecoxib treatment with or without radiation was shown to decrease pChk1 levels in all cell lines tested, and gefitinib treatment was shown to decrease pChk2 levels after radiation in all cell lines.

Both gefitinib (10, 28–31) and celecoxib (32–37) are well-known modulators of apoptosis and cell cycle phases in cancer cells. Therefore, we attempted to determine whether the additive or synergistic clonogenic cytotoxicities and radiation-enhancing effects of combined celecoxib and gefitinib treatments were attributable to additive or synergistic apoptosis induction or cell cycle changes. Combined treatments with celecoxib and gefitinib induced apoptosis in an additive manner in A549 cells and in a synergistic manner in NCI-H460 and VMRC-LCD cells. These results are concordant with the results of clonogenic cytotoxicity assays after treatment with this drug combination. Therefore, the additive or synergistic clonogenic cell deaths that occurred after the combined drug treatment may be, at least partially, caused by apoptotic pathways in the tested cell lines. However, apoptosis induction after celecoxib and gefitinib treatment when combined with radiation does not seem to correlate with results from the clonogenic radiation survival experiments.

Next, we assessed cell cycle changes after the administration of celecoxib and/or gefitinib with or without radiation to determine whether the additive or synergistic clonogenic cytotoxicities and radiation-enhancing effects are related to cell cycle regulation. Celecoxib and/or gefitinib treatments were not shown to cause significant changes in G_2-M phase in the tested cell lines. However, both celecoxib and gefitinib treatment were shown to significantly attenuate radiation-induced G_2-M arrest in NCI-H460 and VMRC-LCD cells and in A549 cells when higher dose of radiation was given. In addition, the combined drug treatment was shown to additively attenuate the radiation-induced G_2-M arrest in these cell lines. These results may imply that celecoxib and gefitinib separately or in combination may act as G_2 checkpoint (or arrest) inhibitors (reviewed in refs. 38, 39). If this is the case, then these drug treatments have the ability to inhibit radiation-induced G_2 checkpoint activation (and G_2 arrest), which would enable more radiation-damaged cells to enter mitosis without appropriate repair and die than would if only radiation treatment had been applied. This hypothesis may constitute a mechanism underlying the radiation-enhancing effects of celecoxib and gefitinib separately or in combination. We then studied the G_2 checkpoint pathway to verify whether the attenuation of radiation-induced G_2-M

arrest is due to G_2 checkpoint deactivation by these drug(s). Interestingly, we found that celecoxib inhibits Chk1 phosphorylation, and gefitinib inhibits Chk2 phosphorylation. Chk1 and Chk2 are well known to play critical roles in radiation-induced G_2 checkpoint activation (and thereby G_2 arrest; ref. 40). Therefore, these data support our hypothesis that celecoxib and gefitinib may be potential G_2 checkpoint inhibitors. However, we did not observe additive or synergistic inhibition of the radiation-activated G_2 checkpoint pathway molecules after the combined celecoxib-gefitinib treatment. These findings and the results of additive G_2-M arrest attenuation by combined drug treatment may imply that celecoxib and gefitinib modulate G_2 checkpoint pathway independently and seem to be concordant with the independent action model applied to combinations of our agents. In addition, the lack of radiation-induced G_2-M arrest attenuation by gefitinib in A549 cells is puzzling, because the inhibition of pChk2 by gefitinib has also been noted in this cell line. There may be complex interactive events between pChk1 and pChk2 inhibition and their downstream molecules after celecoxib and gefitinib treatment to manifest the cooperative attenuation of radiation-induced G_2 arrest. Extensive research on cell cycle regulation by celecoxib and/or gefitinib is warranted.

Taken together, synergistic cell killing after celecoxib and gefitinib treatment may be due to synergistic apoptosis induction, and synergistic radiosensitization by celecoxib or gefitinib may be due to inhibition of radiation-induced G_2 arrest by inhibiting Chk1 or Chk2 activation, respectively. All of these actions seem to occur independently. Therefore, the synergistic clonogenic cell deaths after these three-agent combination treatments may be the combined results of synergistic apoptosis induction after drug combination treatment and synergistic radiosensitizations by attenuation of radiation-induced G_2 arrest after drug-radiation combination treatments.

It is currently unclear in which cancer cells celecoxib and gefitinib treatment can induce synergistic antineoplastic or radiosensitizing effects. Further investigations are needed to determine the factors that govern the additive or synergistic interactions between celecoxib and gefitinib in cancer cells. This knowledge could determine which cancer patients will receive benefits from combined celecoxib and gefitinib treatments with or without radiation.

In conclusion, we suggest that the combined treatment of celecoxib, gefitinib, and radiation can synergistically kill lung cancer cells by additive or synergistic enhancement of apoptosis and by cooperative attenuation of radiation-induced G_2-M arrest. Inhibition of Chk1 and Chk2 activation by celecoxib and gefitinib, respectively, may be responsible for the G_2-M arrest attenuation. The determining factors that cause different manners of apoptosis induction and cell cycle changes in NCI-H460, VMRC-LCD, or A549 cells are not currently clear. Further studies are under way to elucidate the molecular mechanisms underlying the apoptosis and cell cycle regulatory changes observed in this study after the combined drug treatments with or without radiation.

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