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Radiosensitivity Enhancement by Celecoxib, a Cyclooxygenase (COX)-2 Selective Inhibitor, via COX-2-Dependent Cell Cycle Regulation on Human Cancer Cells Expressing Differential COX-2 Levels

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Abstract

To characterize the radiation-enhancing effects on human cancer cells and underlying mechanisms of celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, and to ascertain whether its effects are COX-2 dependent. Clonogenic cytotoxicity assays and radiation survival assays after treatment with celecoxib ± radiation were done on four human cancer cell lines that expressed differential COX-2 levels. Stably COX-2 knocked down or overexpressed cell lines were developed, and clonogenic assays, apoptosis assays, or cell cycle change measurements were conducted after treatment with celecoxib ± radiation. Prostaglandin E₂ (PGE₂) was applied to medium after treatment with celecoxib ± radiation to determine whether the radiation-enhancing effect associated with celecoxib results from reduced generation of prostaglandin. Celecoxib's radiation-enhancing effect was observed in COX-2-expressing A549 and NCI-H460 cells but was not observed in the COX-2 nonexpressing MCF-7 and HCT-116 cells. Celecoxib's radiation-enhancing effects in A549 cells were shown to disappear after the administration of COX-2 knocked down. In contrast, the HCT-116 cells were radiosensitized by celecoxib after being transfected with COX-2 expression vector. The addition of PGE₂ after treatment with celecoxib ± radiation had no significant effects on celecoxib's radiation-enhancing effects in A549 and COX-2 transfected HCT-116 cells. Radiation-induced G₂-M arrest was enhanced and sustained in the COX-2-overexpressing cells compared with that seen in COX-2 low-expressing cells. Celecoxib or NS-398 effected no changes or attenuated radiation-induced G₂-M arrest in the COX-2-overexpressing cells but further enhanced the radiation-induced G₂-M arrest in the COX-2 low-expressing cells. Celecoxib's radiation-enhancing effects seem to occur in a COX-2 expression-dependent manner in the cancer cells. This effect does not seem to be the result of reduced PGE₂ generation. Celecoxib may exert an inhibitory effect on enhanced radiation-induced G₂-M arrest in the COX-2-overexpressing cells, which may allow the arrested

cells to enter mitosis and die after radiation, but may also further enhance radiation-induced G₂-M arrest in the COX-2 low-expressing cells, by virtue of another mechanism. (Cancer Res 2005; 65(20): 9501-9)

Introduction

Cyclooxygenase (COX) is a key enzyme that catalyzes the conversion of arachidonic acid to prostaglandins as well as other prostanoids. Two COX isoforms have, thus far, 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 (1, 2).

Increased COX-2 expression has been observed to occur in a host of tumor types in both humans and animals, and 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 the tumor cells to both chemotherapeutic agents and ionizing radiation (3).

A myriad of studies have been conducted to elucidate the mechanism underlying the antitumor effects associated with COX-2 inhibitors. However, this mechanism has yet to be clearly defined. In addition, debates have raged continuously for a long period over whether the effects of COX-2 selective inhibitors occur in a COX-2 expression-dependent manner in the cancer cells. Several researchers have also reported recently that COX-2 selective inhibitors exert both COX-2-dependent and COX-2-independent antineoplastic effects (4–11).

In terms of the COX-2 dependency of the COX-2 selective inhibitor with regard to radiosensitization, we showed in a previous study that NS-398, a COX-2 selective inhibitor, augmented the effects of radiation in COX-2-overexpressing cells, but this was not found to be true in COX-2 nonexpressing cells (12).

To further clarify this issue, we assessed celecoxib, another COX-2 inhibitor that is currently clinically employed in the treatment of patients with arthritis, on four human cancer cell lines, each of which expressed different COX-2 protein levels. We also constructed and tested COX-2 knocked down cells from A549 cells constitutively expressing high COX-2 levels as well as COX-2-overexpressing cells, which were derived from HCT-116 cells expressing essentially no COX-2, to confirm or disconfirm the COX-2 dependency of the radiation-enhancing effects of the COX-2 selective inhibitor, celecoxib.

Note: Y.K. Shin and J.S. Park contributed equally to this work.

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Materials and Methods

Reagents. Celecoxib was kindly provided by Pharmacia Corp. (Skokie, IL).

Cell culture. A549 human lung adenocarcinoma, MCF-7 human breast carcinoma, NCI-H460 human lung large cell carcinoma, and HCT-116 human colon adenocarcinoma cells were all acquired from the American Type Culture Collection (Manassas, VA) and 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.

Reverse transcription-PCR. Total cellular RNA was extracted with TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. Total RNA (2 µg) was reverse transcribed for 1 hour at 37°C in a reaction mixture that contained 5 units RNase (Amersham Pharmacia Biotech, Piscataway, NJ), 0.5 mmol/L deoxynucleotide triphosphate (Boehringer Mannheim, Indianapolis, IN), 2 µmol/L random hexamer (Stratagene, La Jolla, CA), 1× reverse transcriptase buffer, and 5 units reverse transcriptase (Qiagen, Valencia, CA). We conducted PCR using primers for COX-2 (5'-ATACTAGAGCCCTTCTCTCTGT-3' and 3'-GCA-TACTCTGTTGTGTTCCCTC-5') and glyceraldehyde-3-phosphate dehydrogenase, which was used as an invariant housekeeping gene internal control (5'-CAGGCTGCTTTAACTCTG-3' and 3'-GTCATGAGTCCTCCAGGATAC-5'). Analysis of the resultant PCR products on 1% agarose gel revealed single-band amplification products of the expected sizes.

Immunoblotting. The cells were lysed for 30 minutes at 4°C in radioimmunoprecipitation assay buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA) containing 10 µg/mL aprotinin, 10 µg/mL leupeptin, 1 µg/mL pepstatin, and 100 µg/mL phenylmethylsulfonyl fluoride. The protein concentration of the supernatant in the centrifuged cell lysates was determined using a Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. Proteins (50 µg) were then denatured and fractionated on polyacrylamide gels, which contained SDS, and then transferred to polyvinylidene difluoride membranes after electrophoresis. The filters were then incubated overnight at 4°C in blocking solution (PBS containing 5% nonfat, dried milk and 0.1% Tween 20) followed by 1-hour incubation with anti-COX-2 antibodies. Rabbit polyclonal anti-human COX-2 antibody (Cayman Chemical, Ann Arbor, MI) was used at a dilution of 1:1,000. The filters were washed five times and incubated with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin as a secondary antibody for 1 hour at a 1:5,000 dilution. After five additional washes, the filters were developed with an enhanced chemiluminescence system (Amersham Pharmacia Biotech) and exposed to Hyperfilm enhanced chemiluminescence. The membranes were also probed with anti-actin antibody (Sigma, St. Louis, MO) to normalize the differences between the samples. Quantitation was conducted via video densitometry.

Clonogenic assay for cytotoxicity measurement and radiation survival experiment. Log-phase cells in 75-cm² cell culture flasks were trypsinized and then quantified with a hemocytometer. The cells were then serially diluted to the appropriate concentrations and plated out in triplicate per data point into 25-cm² cell culture flasks. The cells were then permitted to attach for 24 hours at 37°C. Stock celecoxib solutions were constructed via the dissolution of the compound in DMSO (Sigma) and then stored at -20°C. Immediately before the commencement of the experiments, we diluted the stock solutions in DMSO to the appropriate concentrations. To measure cytotoxicity, the cells were exposed to a vehicle (DMSO) or to various celecoxib concentrations for 72 hours. The cells were then rinsed with PBS and allowed to grow in drug-free medium. In the radiation survival experiment, the cells were exposed to a vehicle (DMSO) or to various celecoxib concentrations for 4 hours. The cells were then irradiated with graded doses of γ-rays using the Gammacell 3000 Elan system (MDS Nordion, Inc., Ontario, Canada), at a dose rate of 10.7 Gy/min, as determined by thermoluminescence dosimetry for the specific system employed. After an additional 68 hours of incubation in medium containing either the vehicle or the drug, the cells were rinsed with PBS, and drug-free

medium was added. The final DMSO concentration was adjusted to 0.2% in all flasks. The cells were then maintained at 37°C for 6 to 8 days to allow for the formation of colonies and then stained with 0.5% crystal violet (Sigma) in absolute methanol. The colonies were counted visually with a cutoff value of 50 viable cells. The surviving fraction was then calculated as follows: mean number of colonies / (number of cells inoculated × plating efficiency), where plating efficiency is defined as the mean number of colonies / number of cells inoculated for untreated controls. Surviving fractions in the cells exposed to radiation plus celecoxib were normalized by dividing by the surviving fraction obtained for celecoxib alone. We then calculated the dose enhancement ratio (DER) as the dose (Gy) for the radiation plus vehicle cells divided by the dose (Gy) for radiation plus celecoxib (normalized for drug toxicity) at a surviving fraction of 0.1. Error bars were also calculated as SE via the pooling of the results of three independent experiments.

Development of stably COX-2 knocked down cells by RNA interference. We designed and synthesized complementary oligonucleotides against human COX-2, containing 5' single-strand overhangs for ligation into the pSilenCircle (Allele Biotechnology, San Diego, CA) vector for three small interfering RNA (siRNA) target candidate sequences. One candidate target sequence (5'-acaccGAACGTTTCGACTGAACTGTTgcttgaa-ACAGTTCAGTCGAACGTTTc-3' and 3'-aaaaaGAACGTTTCGACTGAACTGTT-tcaagcaaACAGTTCAGTCGAACGTTTc-5') was shown to work properly by the results of a gene knockdown assay, which employed reverse transcription-PCR (RT-PCR) and Western blotting. The negative control sequence was adopted from the general negative sequence (Ambion, Inc., Austin, TX). The oligonucleotides were then annealed and ligated into the pSilenCircle vector. Transfection was conducted with LipofectAMINE reagent (Invitrogen) in accordance with the manufacturer's instructions. Positive transfectants were selected in medium, which contained 500 units/mL G418 (Life Technologies). All cell lines were derived from individual colonies via cloning cylinders.

Development of stably transfected cells with COX-2 cDNA. The human COX-2 expression vector (pSG5-COX-2 plasmid, kindly provided by Dr. S.J. Lim, National Cancer Center, Goyang-si, Gyeonggi-do, South Korea), which harbors a full-length COX-2 cDNA, was transfected into HCT-116 cells that have been grown in six-well tissue culture plates at a density of 3×10^5 cells per 2 mL medium. The transfection and selection of the positive transfectant were conducted as described above using 200 µg/mL hygromycin (Life Technologies).

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-cm² flasks for the determination of each data point. After 24 hours, the cells were exposed to the appropriate concentrations of celecoxib or vehicle (DMSO) for 4 hours and then exposed to graded doses of γ-rays. After an additional 20, 44, or 68 hours of incubation in medium, which contained either the drug or the vehicle, the cells were trypsinized (retaining all floating cells), fixed with 75% ethanol at -20°C overnight, and then incubated at room temperature for 3 hours with 10 µg/mL propidium iodide (Sigma) and 5 µg/mL RNase A (Amresco, Solon, OH). The number of cells at each cell cycle as well as the cells that have undergone apoptosis (sub-G₁) was evaluated with the FACSCalibur system (Becton Dickinson, San Jose, CA). Error bars were also calculated as SE by the pooling of the results of three independent experiments. The same samples were then mounted on slide glasses and were reanalyzed under fluorescent microscopy.

Statistical analysis. Data were expressed as means ± SE and then analyzed with regard to statistical significance using ANOVA followed by Scheffé's test for multiple comparisons. $P < 0.05$ was considered to be significant.

Results

Cytotoxicity of celecoxib on cancer cells. A549 cells exhibited a high constitutive COX-2 protein expression level, and the NCI-H460 cells expressed relatively low COX-2 levels. The MCF-7 and HCT-116 cells manifested no detectable expression of the COX-2 protein (Fig. 1). To assess celecoxib's cytotoxicity on cancer cells,

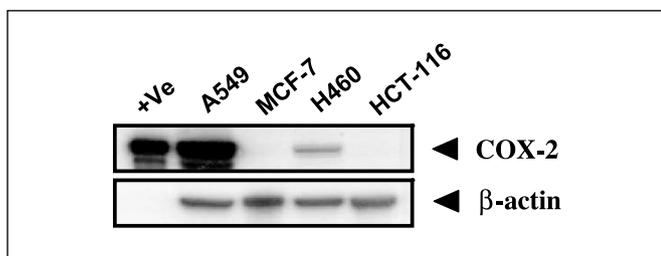


Figure 1. Western blot analysis for COX-2 expression in A549, MCF-7, NCI-H460, and HCT-116 cells. +Ve, positive control of COX-2 (ovine COX-2 electrophoresis standard). A549 cells showed high level of constitutive COX-2 protein expression, NCI-H460 cells expressed low level of COX-2, and MCF-7 and HCT-116 cells exhibited no constitutive COX-2 expression.

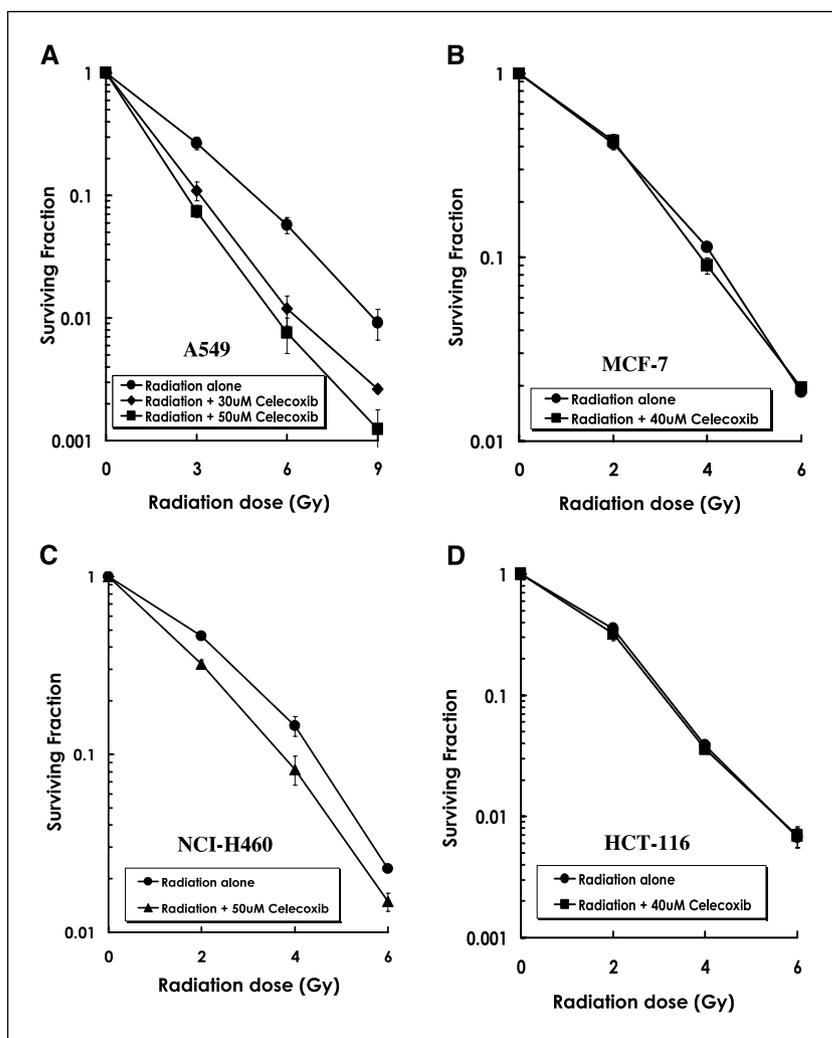
the cells were exposed to various celecoxib dosages for 72 hours and then permitted to form colonies in drug-free medium. Clonogenic cytotoxicity values for celecoxib in the different cancer cells were not found to differ significantly according to COX-2 expression levels in these cells (data not shown).

Celecoxib's effects on the radiosensitivity of various cancer cells. To assess and characterize the radiation-enhancing effects of celecoxib, the cells were exposed to graded doses of γ -radiation either with or without IC_{50} concentrations of celecoxib treatments

for 72 hours. These cells were all then permitted to form colonies in drug-free medium. Radiation was administered 4 hours after the start of drug treatment. Celecoxib was determined to augment the effects of radiation in the A549 and NCI-H460 cells, and the DER values were determined to be 1.6 to 1.9 and 1.2 at a surviving fraction of 0.1, respectively (Fig. 2A and C). However, celecoxib exerted no radiation-enhancing effects in the MCF-7 and HCT-116 cells (Fig. 2B and D). Various celecoxib concentrations (20-60 μ mol/L) were tested in the MCF-7 and HCT-116 cells, but we were unable to detect any enhancement of radiation effects (data not shown).

Celecoxib's effects on the clonogenic survival of A549-mock and A549-COX-2 knockdown cells. To determine whether celecoxib's radiation-enhancing effects in A549 cells occur in a COX-2-dependent manner, a stably COX-2 knocked down cell line (AS), which employed the siRNA expression vector for COX-2, and its mock control cell line (AN), were developed from A549 cells, and we conducted a series of clonogenic assays with these cells. The AS line exhibited >90% knockdown of COX-2 mRNA and protein expression compared with its parent cells, whereas we witnessed no changes in COX-2 expression in the AN cells (Fig. 3A). Prostaglandin E₂ (PGE₂) generation in the AN cells was comparable with that observed in the A549 parent cells. However, PGE₂ generation in the AS cells was noted to have decreased to 1% that observed in the parent or the AN cells (Table 1).

Figure 2. Clonogenic survival curves for radiation + celecoxib in A549 (A), MCF-7 (B), NCI-H460 (C), and HCT-116 (D) cells. Cells attached to the flasks were treated with celecoxib for 4 hours, irradiated, rinsed after another 68 hours, and allowed to form colonies in drug-free medium. Surviving fractions for radiation + celecoxib were normalized by dividing by the surviving fraction for celecoxib only. A, ●, radiation + vehicle (DMSO) treatment; ◆, radiation + 30 μ mol/L celecoxib; ■, radiation + 50 μ mol/L celecoxib in A549 cells. B, ●, radiation + vehicle (DMSO) treatment; ■, radiation + 40 μ mol/L celecoxib in MCF-7 cells. C, ●, radiation + vehicle (DMSO) treatment; ▲, radiation + 50 μ mol/L celecoxib in NCI-H460 cells. D, ●, radiation + vehicle (DMSO) treatment; ■, radiation + 40 μ mol/L celecoxib in HCT-116 cells. Bars, SE of three independent experiments in triplicate. COX-2-overexpressing A549 cells exhibited dose-dependent radiation-enhancing effects as the result of celecoxib treatment (A), and COX-2-expressing NCI-H460 cells exhibited lesser degrees of radiation-enhancing effects than did the A549 cells (C). By way of contrast, COX-2 nonexpressing MCF-7 (B) and HCT-116 (D) cells exhibited no radiation-enhancing effects as the result of treatment with this drug.



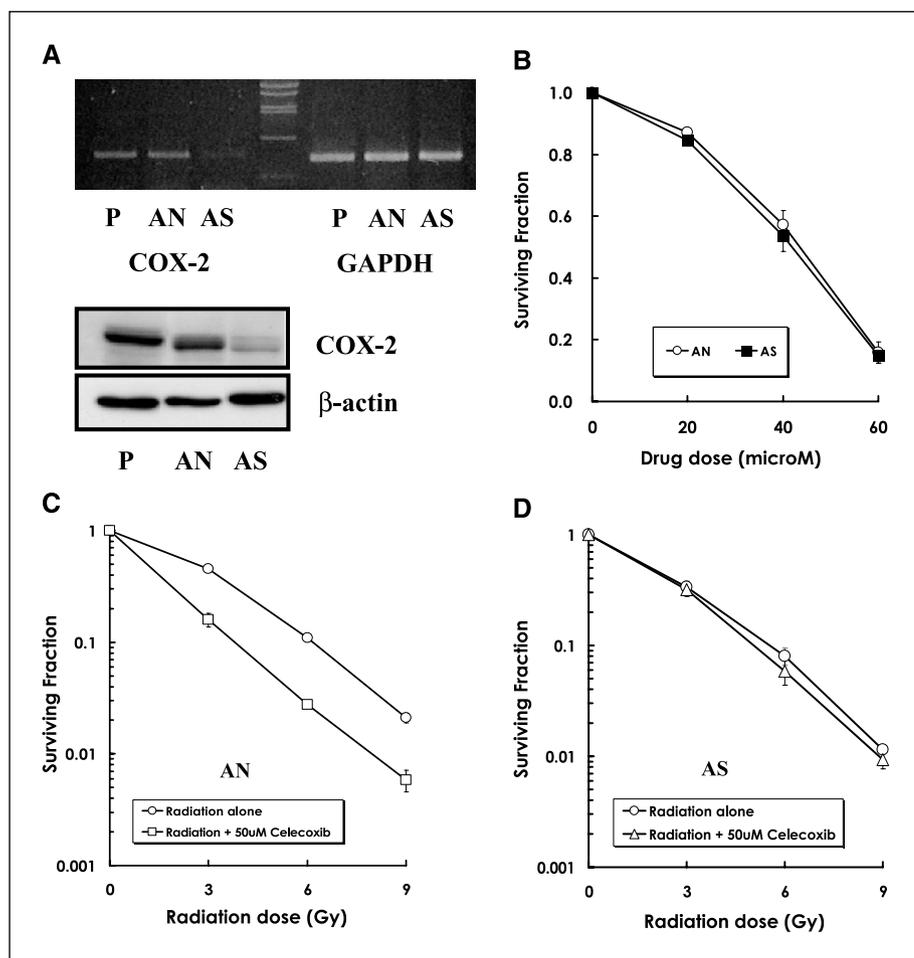


Figure 3. Clonogenic cytotoxicity and radiation survival curves for radiation \pm celecoxib in the stably COX-2 knocked down cells developed from A549 cells via transfection with COX-2 siRNA-expressing vector. *A*, results of RT-PCR (top) and Western blots (bottom) for COX-2 expression. *P*, A549 parent cells; *AN*, COX-2 knocked down A549 cells; *AS*, mock control A549 cells transfected with a general negative sequence harboring vectors. *B*, clonogenic cytotoxicity curves for celecoxib in AN and AS cells. \circ , AN; \blacksquare , AS. *C*, clonogenic survival curves for radiation + celecoxib in AN. \circ , radiation + vehicle (DMSO) treatment; \square , radiation + 50 μ M/L celecoxib. *D*, clonogenic survival curves for radiation + celecoxib in AS. \circ , radiation + vehicle (DMSO) treatment; \triangle , radiation + 50 μ M/L celecoxib. Bars, SE of three independent experiments in triplicate. AN exhibited almost identical COX-2 expression with that of the A549 parent cells, and AS manifested >90% reduction of COX-2 mRNA and protein expression (*A*). We detected no differences in clonogenic cytotoxicity between these two cell lines (*B*). Celecoxib sensitized AN cells to radiation, but this effect disappeared in the AS cells (*C* and *D*).

Celecoxib-associated cytotoxicity was similar in both cell lines (Fig. 3*B*). Celecoxib-associated radiation-enhancing effects were observed in the AN cells, but these effects disappeared in the AS cells (Fig. 3*C* and *D*).

Celecoxib's effects on the clonogenic survival of HCT-116-mock and HCT-116-COX-2 overexpressed cells. To determine whether the lack of celecoxib's radiation-enhancing effects in the HCT-116 cells could be reversed via the induction of COX-2 expression in these cells, we constructed both a stably COX-2 transfected HCT-116 cell line (HCT-116-COX-2) and a mock control cell line (HCT-116-mock). We then conducted a series of clonogenic assays on both of these cell lines. The HCT-116-COX-2 cells were observed to express significant quantities of the COX-2 protein, comparable with that seen in the A549 cells, and this was a functional protein, whereas the HCT-116-mock cell line was shown to express no COX-2 (Fig. 4*A*; Table 1). The HCT-116-COX-2 cells were more sensitive to celecoxib treatment than were the HCT-116-mock cells (Fig. 4*B*). The HCT-116-mock cell line manifested no detectable radiation-enhancing effects as the result of celecoxib exposure. However, HCT-116-COX-2 cells evidenced a mild radiation-enhancing effect on exposure to this drug (Fig. 4*C* and *D*).

Effects of prostaglandin E₂ addition after radiation treatment combined with celecoxib in the A549 and HCT-116-COX-2 cells. To determine whether the radiation-enhancing effects of celecoxib in the A549 and HCT-116-COX-2 cells were attributable to the reduced prostaglandin generation as the result of treatment with this drug, PGE₂, a primary product of the COX-2 enzyme, was

added to the cells after the administration of celecoxib and/or radiation treatment, after which we conducted a set of clonogenic radiation survival experiments. After testing a variety of PGE₂ concentrations (10 nmol/L-10 μ M/L) for the clonogenicity of A549 and HCT-116-COX-2 cells, 500 nmol/L PGE₂ (equals 176.3 ng/mL), which is equivalent to the maximum inducible concentration in the A549 cells (refs. 13-18; Table 1), was added to the medium to supply a sufficient amount of PGE₂ but not to exceed the physiologic range. This PGE₂ concentration was not found to affect the clonogenic survival of the A549 and HCT-116-COX-2 cells when administered alone. PGE₂ administration was also shown not to affect the clonogenic radiation survival characteristics of these cells, nor did it detectably affect the radiation-enhancing effects associated with celecoxib treatment (data not shown).

Effects of celecoxib on apoptotic induction in AN, AS, HCT-116-mock, and HCT-116-COX-2 cells. We then attempted to determine whether the radiation-enhancing effects of celecoxib in the AN and HCT-116-COX-2 cells but not in the AS and HCT-116-mock cells was attributable to differences in apoptotic induction by this drug in these cells. In brief, the cells were exposed to celecoxib for up to 72 hours, either with or without radiation, and the fraction of sub-G₁ was then calculated to measure the rate of apoptosis after flow cytometric analysis. The apoptosis induction rates associated with treatment with 50 or 40 μ M/L celecoxib alone (for AN and AS or HCT-116-COX-2 and mock, respectively) were relatively low in all of the tested cell lines. Apoptotic induction rates after radiation and celecoxib treatment did not

seem to have undergone a synergistic increase in any of the cells when compared with the values observed after the administration of each treatment separately (Table 2).

Celecoxib's effects on cell cycle regulation in the AN, AS, HCT-116-mock, and HCT-116-COX-2 cells. To determine whether the radiation-enhancing effects of celecoxib in the AN and HCT-116-COX-2 cells but not in the AS and HCT-116-mock cells was related to cell cycle regulation as a result of treatment with this drug, the number of cells in each cell cycle phase was determined via flow cytometric analyses after the administration of celecoxib treatment, either with or without radiation in these cell lines, for up to 72 hours. All four of the tested cell lines exhibited significantly increased rates of G₀-G₁-phase cells after the administration of 50 or 40 μmol/L celecoxib (for AN and AS or HCT-116-COX-2 and mock cells, respectively), and we determined there to be no differences resulting from differential COX-2 expression levels in these cells (data not shown). AN and HCT-116-COX-2 manifested significantly higher and sustained rates of G₂-M-phase cells compared with what was observed with the AS and HCT-116-mock cells, respectively, when treated with 9 Gy (AN and AS) or 6 Gy (HCT-116-mock and HCT-116-COX-2) of radiation alone (Fig. 5). Celecoxib treatment combined with radiation in the AS and HCT-116-mock cells resulted in significantly increased and sustained rates of G₂-M-phase cells compared with what was observed as the result of radiation administered alone in each cell lines, whereas combined treatment in the AN cells resulted in only minor differences in the rate of G₂-M cells compared with what was seen with radiation alone. Combined treatment in the HCT-116-COX-2 cells resulted in a reduction in the amount of G₂-M-phase cells compared with what was observed with radiation alone (Fig. 5A and B). We also tested another COX-2 selective inhibitor, NS-398, to ascertain whether the effects on cell cycle changes observed in conjunction with celecoxib treatment were similar to the effects reported in association with other COX-2 selective inhibitors. Combined treatment with NS-398 (at a IC₅₀ concentration for clonogenic death after 72 hours of drug treatment) and radiation in HCT-116-mock cells was reported to both increase

and sustain the rate at which cells were found in the G₂-M phase compared with what was observed after the administration of radiation only. However, we noted only minor changes in HCT-116-COX-2 cells (Fig. 5C). This is consistent with the results obtained using celecoxib, although the reduction in the amount of cells found in the G₂-M phase was not observed in this experimental setting after the treatment of our experimental cells with a combination of the drug and radiation.

Discussion

Although many results have been reported with regard to the radiosensitizing effects exerted by COX-2 selective inhibitors on a variety of cancer cells (12, 19–26), the mechanisms underlying these effects have yet to be clearly understood. There is also little available data, at this point, regarding COX-2-dependent radiosensitization by COX-2 selective inhibitors. In our previous study, we determined that NS-398 could enhance the radiosensitivity of only COX-2-overexpressing RIE-S and NCI-H460 cells (12). In the current study, we evaluated four human cancer cell lines with a variety of pathologic cell types culled from several different organs and determined that celecoxib, another clinically available COX-2 selective inhibitor, also enhanced the effect of radiation only in COX-2-overexpressing A549 and NCI-H460 cells and did not enhance the effect of radiation in COX-2 nonexpressing MCF-7 and HCT-116 cells. However, the cytotoxicities of celecoxib alone were not shown to be dependent on COX-2 expression levels. This result is consistent with the results from the previous study. Moreover, we studied celecoxib-associated radiosensitization in COX-2 knocked down A549 and COX-2 overexpressed HCT-116 cells to confirm the COX-2 dependency inherent to the radiosensitization effects of this drug. We discovered that the previously described radiation-enhancing effects of celecoxib disappeared when the COX-2 was knocked down in the A549 cells by RNA interference, whereas the lack of radiosensitization as the result of celecoxib treatment in the COX-2-negative HCT-116 cells was reversed after the transfection of COX-2 cDNA in these cells. According to the results of our previous and current studies, it can be inferred that COX-2 selective inhibitors primarily affect the sensitization of COX-2-expressing cells to radiation and do not tend to confer this sensitivity to COX-2 nonexpressing cells. This may, in fact, constitute a common feature of COX-2 inhibitors. Therefore, it would be reasonable to surmise that radiosensitization as the result of treatment with COX-2 selective inhibitors occurs via a COX-2 protein-dependent mechanism in the cells.

A549 cells, which express high COX-2 levels, exhibited higher DER values as the result of celecoxib treatment than were observed in NCI-H460 cells, which express relatively low COX-2 levels. However, the DER values seen as the result of celecoxib treatment in the HCT-116-COX-2 cells were lower than that in the A549 cells despite the comparable COX-2 expression levels in these two cell lines. This suggests that the COX-2-dependent radiation-enhancing effects associated with celecoxib are dependent on the presence of COX-2 protein in the cells but are not dependent on the levels of COX-2 expression, and the DER values of the COX-2 selective inhibitors may be determined principally by other factors in the cells.

We reported in a previous study that the mechanism underlying the radiation-enhancing effects of NS-398 in RIE-S and NCI-H460 cells may be attributable to the enhancement of radiation-induced apoptosis as the result of treatment with this drug (12). However, only a minor amount of apoptotic induction was noted as the

Table 1. PGE₂ production in AN, AS, HCT-116-mock, and HCT-116-COX-2 cells before and after stimulation by adding arachidonic acid into medium for 24 hours

Cells	PGE ₂ concentration (ng/million cells)	SE*
Unstimulated		
A549 parent	5.2	0.4
AN	5.0	0.5
AS	0.06	0.01
HCT-116-parent	0.004	0.0008
HCT-116-mock	0.006	0.0009
HCT-116-COX-2	10.3	0.9
Stimulated by 10 μmol/L arachidonic acid		
A549 parent	8.4	0.5
AN	7.5	2.4
AS	0.4	0.08
HCT-116-parent	0.5	0.01
HCT-116-mock	0.2	0.02
HCT-116-COX-2	52.5	2.3

*SE from two independent experiments.

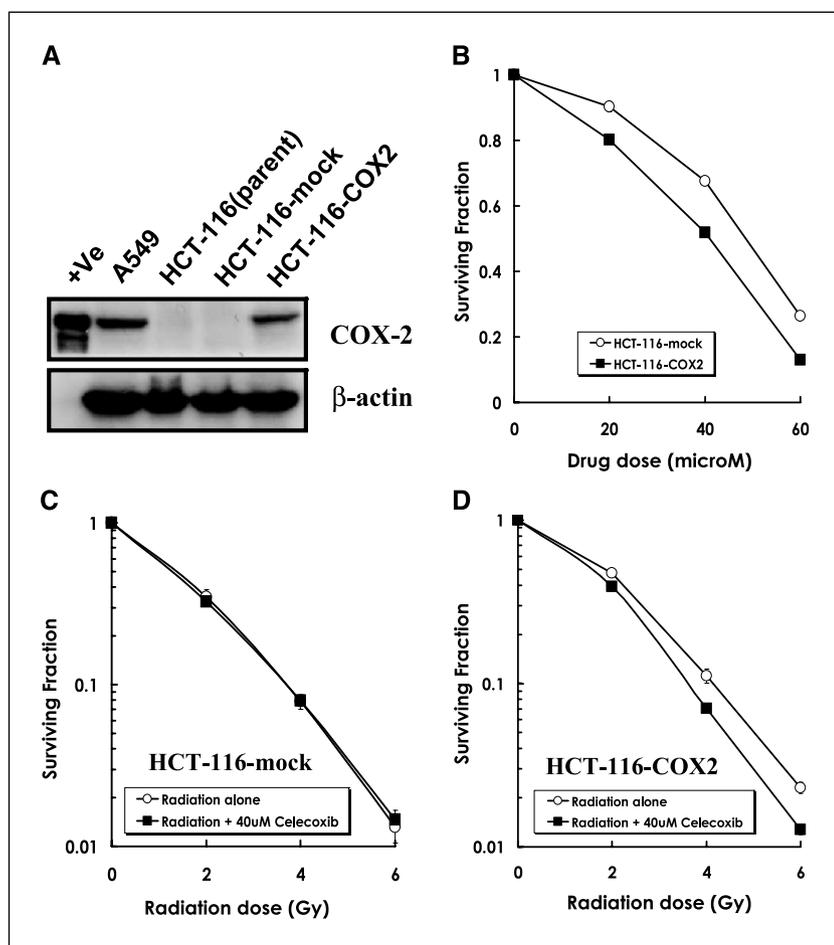


Figure 4. Clonogenic cytotoxicity and radiation survival curves for radiation \pm celecoxib in the stably COX-2 overexpressed cells developed from the HCT-116 cells via the transfection of COX-2 expression vector. *A*, Western blots for COX-2 expression. +Ve, positive COX-2 control. *B*, clonogenic cytotoxicity curves for celecoxib in the HCT-116-mock (\circ) and HCT-116-COX-2 (\blacksquare) cells. *C*, clonogenic survival curves for radiation + celecoxib in HCT-116-mock cells. \circ , radiation + vehicle (DMSO) treatment; \blacksquare , radiation + 40 μ mol/L celecoxib. *D*, clonogenic survival curves for radiation + celecoxib in HCT-116-COX-2 cells. \circ , radiation + vehicle (DMSO) treatment; \blacksquare , radiation + 40 μ mol/L celecoxib. Bars, SE of three independent experiments in triplicate. HCT-116-mock cells exhibited no COX-2 expression, whereas the HCT-116-COX-2 cells expressed an amount of COX-2 comparable with that of A549 cells (*A*). HCT-116-COX-2 cells were determined to be more sensitive to celecoxib than were the HCT-116-mock cells (*B*). HCT-116-mock cells exhibited no radiation-enhancing effects, whereas HCT-116-COX-2 cells exhibited a mild radiation-enhancing effect as the result of celecoxib treatment (*C* and *D*).

result of celecoxib treatment, with or without radiation, in the AN, AS, HCT-116-mock, and HCT-116-COX-2 cells evaluated in the current study. In addition, the degree to which apoptosis was induced by celecoxib and radiation was not determined to be synergistically increased in the COX-2-overexpressing AN and HCT-116-COX-2 cells. The reasons for this difference between the results of the previous and current study will require further study before they can clearly elucidated, but different drug incubation times (24 hours for NS-398 versus 72 hours for celecoxib) may be partially responsible for radiosensitization occurring as the result of different mechanisms; treatment with a high concentration of COX-2 selective inhibitors for a short time may affect the sensitization of the cells via a pathway involving apoptosis. By way of contrast, treatment with low concentrations of COX-2 selective inhibitors for a prolonged period may exert an effect via completely different mechanisms, such as cell cycle modulation. These will be discussed later in this section.

After we had confirmed the COX-2-dependent radiosensitization by COX-2 selective inhibitors occurring in the current study, we became curious as to whether the radiosensitizing effects observed in the COX-2 expressing cells was perhaps attributable to an attenuation of prostaglandin generation as the result of treatment with these drugs. Reports regarding prostaglandin treatment for the modulation of cancer cell radiosensitivity have not been consistent. PGE₁, PGE₂, and their analogues have been shown to protect normal tissues from radiation but not cancer cells (27, 28). Some prostaglandins have been shown, in fact, to potentiate radiation

toxicity in cancer cells (29, 30). To characterize the relationship between attenuated prostaglandin generation and radiation sensitivity or radiosensitization of cells as the result of COX-2 selective inhibitors, we treated cells with PGE₂, a primary product of COX-2 in the tested cells (31), at a concentration that was believed to be sufficiently high for the verifiable emergence of its effects but within a physiologic range that would preclude its own cytotoxic effects or unexpected nonphysiologic effects in the cells. The addition of PGE₂ was determined neither to alter radiation survival rates nor to reverse celecoxib's radiation-enhancing effects on A549 and HCT-116-COX-2 cells. This result suggests that physiologic PGE₂ concentrations have no effect on radiation survival rates of cancer cells and also that celecoxib-induced radiosensitization may not constitute the result of attenuated prostaglandin generation. Therefore, signals for radiation-enhancing effects resultant from treatment with COX-2 selective inhibitors may be mediated by the COX-2 protein itself or by substrates or enzymes in the upstream regions of this protein but not by its end product.

COX-2 selective inhibitors are well known to modulate the cell cycles of both normal and cancer cells. COX-2 selective inhibitors have been shown to primarily induce G₀-G₁ arrest (4, 9, 32-36); however, some drugs have been shown, under some conditions, to induce G₂-M arrest (22, 37, 38). Several researchers did cell cycle analyses to investigate the mechanisms underlying the radiation-enhancing effects of the COX-2 selective inhibitors. However, most of this research involved the measurements of cell cycle changes after treatment with COX-2 selective inhibitor in the absence of radiation,

or involved only one cell line, without regard to the COX-2 expression inherent to that line (21, 22, 38). Therefore, there have been no previous comparative analyses conducted regarding cell cycle modulation after combined treatment of COX-2 selective inhibitors with radiation according to differences in COX-2 expression in the cells. In the current study, we measured changes in the amount of cells in each cell cycle phase in AN, AS, HCT-116-mock, and HCT-116-COX-2 cells, after celecoxib treatment either with or without radiation, to further determine the mechanisms underlying COX-2-dependent radiosensitization as the result of treatment with COX-2 selective inhibitors. Radiation treatment alone was shown to induce significant G₂-M arrest, which is a well-known phenomenon (reviewed in ref. 39). However, this radiation-induced G₂-M arrest was enhanced and sustained to a greater degree in the COX-2-overexpressing cells than in their COX-2 low-expressing counterparts. This implies that COX-2 expression may be associated with the regulation of G₂-M phase, such as the G₂-M checkpoint, after treatment with DNA-damaging agents, including radiation.

Celecoxib treatment alone was shown to induce G₁ arrest in all four of the tested cell lines, and this has been already shown in many previous reports and was an expected result. However, celecoxib treatment combined with radiation treatment was shown to induce different types of cell cycle changes in the cells. Celecoxib treatment was shown to attenuate radiation-induced G₂-M arrest in the COX-2-overexpressing HCT-116-COX-2 cells or resulted in only minor changes in the duration of radiation-induced G₂-M arrest in the COX-2-overexpressing AN cells. By way of contrast, celecoxib treatment further enhanced radiation-induced G₂-M arrest in the COX-2 low-expressing cells. Experiments with NS-398 yielded similar results, although the attenuation of radiation-induced G₂-M arrest in the COX-2-overexpressing HCT-116-COX-2 cells, which was observed as the result of celecoxib treatment in the same cells, was

Treatment group	24 h	48 h	72 h
AN cells			
Control	0.47 ± 0.14*	0.73 ± 0.08	1.19 ± 0.10
Celecoxib 50 µmol/L	0.44 ± 0.15	0.51 ± 0.01	3.47 ± 0.05
Radiation 9 Gy	1.16 ± 0.28	2.32 ± 0.07	4.00 ± 0.31
Radiation + celecoxib	1.10 ± 0.21	1.80 ± 0.39	3.88 ± 0.80
AS cells			
Control	0.80 ± 0.08	1.34 ± 0.16	2.12 ± 0.11
Celecoxib 50 µmol/L	0.94 ± 0.11	1.23 ± 0.20	2.73 ± 0.40
Radiation 9Gy	0.86 ± 0.15	3.67 ± 1.24	4.94 ± 1.80
Radiation + celecoxib	1.29 ± 0.22	2.14 ± 0.02	3.55 ± 0.11
HCT-116-mock cells			
Control	1.77 ± 0.43	1.82 ± 0.59	1.11 ± 0.04
Celecoxib 40 µmol/L	1.99 ± 0.54	1.70 ± 0.24	1.34 ± 0.26
Radiation 6Gy	1.86 ± 0.94	2.23 ± 0.45	3.85 ± 0.28
Radiation + celecoxib	2.18 ± 0.78	2.33 ± 0.51	2.78 ± 0.16
HCT-116-COX-2 cells			
Control	2.62 ± 0.92	2.30 ± 0.32	2.80 ± 0.23
Celecoxib 40 µmol/L	3.67 ± 1.13	4.43 ± 0.30	4.22 ± 1.02
Radiation 6Gy	4.43 ± 0.54	6.54 ± 0.98	12.85 ± 3.11
Radiation + celecoxib	5.39 ± 0.98	5.75 ± 0.47	7.87 ± 0.58

*Mean ± SE from three independent experiments.

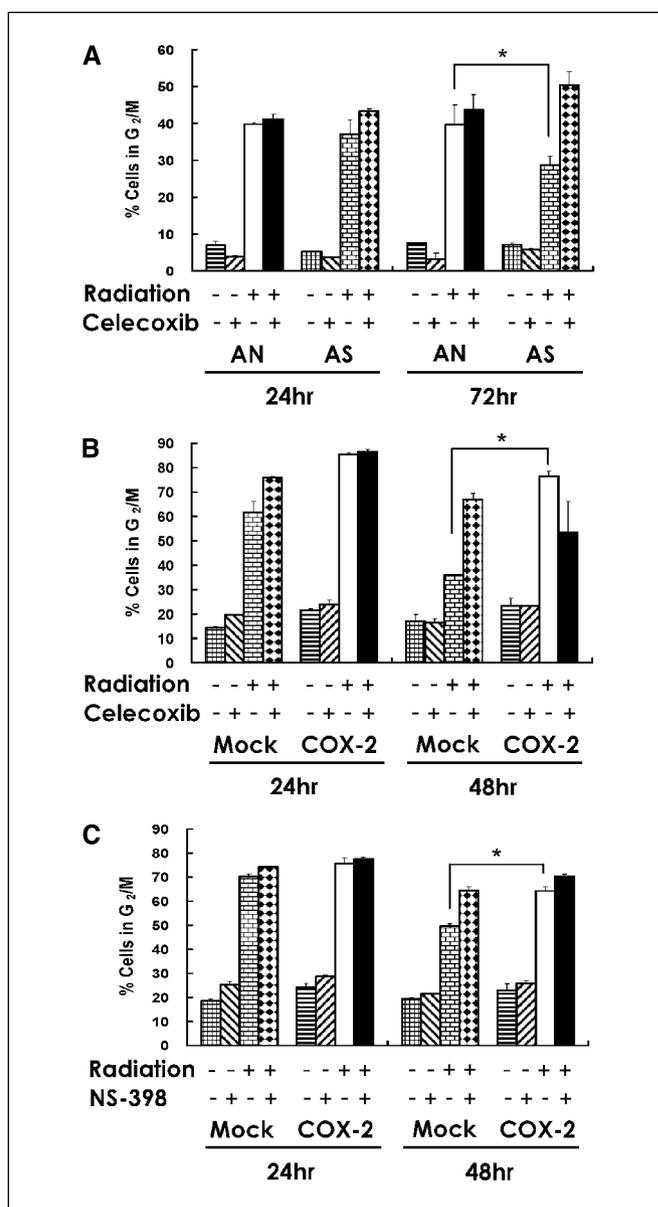


Figure 5. Cell cycle analyses after celecoxib ± radiation treatment in the AN and AS (A) and HCT-116-mock and HCT-116-COX-2 (B) cells or after NS-398 ± radiation treatment in the HCT-116-mock and HCT-116-COX-2 cells (C). The attached cells were exposed to appropriate concentrations of celecoxib, NS-398, or vehicle (DMSO) for 4 hours, irradiated (9 Gy for AN and AS or 6 Gy for HCT-116-mock and COX-2), harvested, and fixed with 75% ethanol after an additional 20, 44, or 68 hours of incubation in medium containing the relevant drug. The cells were then stained with propidium iodide, and the number of cells at different cell cycle phases was measured by flow cytometry. Bars, SE of three independent experiments. *, *P* < 0.05. Radiation treatment alone manifested higher and sustained rates of G₂-M-phase cells in the COX-2-overexpressing AN and HCT-116-COX-2 cells compared with what was observed in the COX-2 low-expressing AS and HCT-116-mock cells. Celecoxib or NS-398 effected no changes (A and C) or decreased (B) the rates of G₂-M-phase cells after radiation treatment in AN or HCT-116-COX-2 cells, whereas these drugs were shown to further increase the rates of G₂-M-phase cells after radiation treatment in the AS cells (A) or HCT-116-mock cells (B and C).

not observed in conjunction with this drug. These results indicate that COX-2 selective inhibitors initiate dual COX-2-dependent actions on cell cycle regulation after radiation treatment. To explain this unique phenomenon, we hypothesized that COX-2 overexpression in these cells both promotes and sustains the activation of G₂-M checkpoint after radiation, thereby enhancing radiation-induced

G₂-M arrest to protect the cells against radiation, and that COX-2 selective inhibitors inhibit this G₂-M checkpoint promotion in COX-2-overexpressing cells. The COX-2-overexpressing cells may then induce higher and sustained degrees of G₂-M arrest after radiation compared with that in the COX-2 nonexpressing or low-expressing cells, and COX-2 selective inhibitors may inhibit this prolonged G₂-M arrest in COX-2-overexpressing cells. In such a case, more radiation-damaged cells will then enter mitosis without appropriate repair, and die, than would if only radiation treatment had been applied. The number of cells in the G₂-M phase may decrease, then, as the result of increased cell death, or may merely fail to increase, as flow cytometric analysis after propidium iodide staining alone cannot distinguish the cells in the G₂ phase from those in mitotic phase. This hypothesis may constitute a mechanism underlying the radiation-enhancing effects of COX-2 selective inhibitors in COX-2-overexpressing cells. In contrast, COX-2 selective inhibitors may also exert a radiation-induced G₂-M arrest enhancement effect in COX-2 nonexpressing or low-expressing cells by a yet unknown mechanism. If this is the case, then the number of cells in the G₂-M phase could be increased as the result of the combined treatment of these drugs with radiation. The relationship between this radiation-induced G₂-M arrest enhancement effect and the lack of radiation-enhancing effect observed when these drugs are administered to COX-2 nonexpressing or low-expressing cells remains a matter of some controversy and requires further investigation, including separate analyses of cells in G₂ or mitotic phase. These cell cycle-regulating effects may constitute a common feature of COX-2 selective inhibitors, as studies of two distinct COX-2 selective inhibitors (celecoxib and NS-398) yielded almost identical results. To the best of our knowledge, the COX-2-dependent dual action of COX-2 selective

inhibitors on irradiated cells has not yet been reported. Further studies are currently under way to define the optimum treatment schedule of COX-2 selective inhibitors relative to radiation, to maximally induce attenuation of radiation-induced G₂-M arrest with these drugs, and to elucidate the molecular mechanisms underlying these unique properties of COX-2 selective inhibitors as well as their relationships with radiosensitization.

In summary, we conclude that the radiation-enhancing effects associated with celecoxib, a COX-2 selective inhibitor, occur in a COX-2 expression-dependent manner in the cells. These effects do not seem to originate from attenuated prostaglandin generation by celecoxib, nor do they seem to be the result of increased radiation-induced apoptosis. Celecoxib (or NS-398) exhibited a dual mode of action on cell cycle regulation after combined treatment with radiation; no changes or attenuated radiation-induced G₂-M arrest were observed in the COX-2-overexpressing cells, and this effect may allow the arrested cells to enter mitosis and die after radiation. By way of contrast, celecoxib further enhanced radiation-induced G₂-M arrest in the COX-2 low-expressing cells. The molecular mechanisms underlying these celecoxib-associated effects will require further elucidation, and these results may bear some clinical importance with regard to potential applications of celecoxib in cancer patients undergoing radiotherapy.

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