Enhancement of Intrinsic Tumor Cell Radiosensitivity Induced by a Selective Cyclooxygenase-2 Inhibitor

Cordula Petersen, Sven Petersen, Luka Milas, Frederick F. Lang, and Philip J. Tofilon[1]

Departments of Experimental Radiation Oncology [C. P., S. P., L. M., P. J. T.] and Neurosurgery [F. F. L., P. J. T.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

The antitumor effects of the selective cyclooxygenase (COX)-2 inhibitor SC-236 alone and in combination with radiation were investigated using the human glioma cell line U251 grown in monolayer culture and as tumor xenografts. On the basis of Western and Northern blot analyses, these cells express COX-2 protein and mRNA to levels similar to those in the human colon carcinoma cell line HT29. Treatment of U251 cells in monolayer culture with 50 μM SC-236 resulted in a time-dependent decrease in cell survival as determined by a clonogenic assay. The cell death induced by SC-236 was associated with apoptosis and the detachment of cells from the monolayer. After 2 days of drug treatment, the cells that remained attached were exposed to graded doses of radiation, and the clonogenic assay was performed. Comparison of the survival curves for drug-treated and untreated cultures revealed that SC-236 enhanced radiation-induced cell death. In these combination studies, SC-236 treatment resulted in a dose-enhancement factor of 1.4 at a surviving fraction of 0.1, with the surviving fraction at 2 Gy (SF2) reduced from 0.61 to 0.31. These data indicate that *in vitro* SC-236 induces U251 apoptotic cell death and enhances the radiosensitivity of the surviving cells. To extend these investigations to an *in vivo* situation, U251 glioma cells were grown as tumor xenografts in the hind leg of nude mice, and SC-236 was administered in drinking water. SC-236 alone slowed tumor growth rate, and when administered in combination with local irradiation, SC-236 caused a greater than additive increase in tumor growth delay. These *in vitro* and *in vivo* results suggest that the selective inhibition of COX-2 combined with radiation has potential as a cancer treatment.

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[1] To whom requests for reprints should be addressed, at University of Texas M. D. Anderson Cancer, Department of Experimental Radiation Oncology, 1515 Holcombe Boulevard, Box 66, Houston, Texas 77030. Phone: (713) 792-3424; Fax: (713) 794-5369; E-mail: ptofilon@notes.mdacc.tmc.edu.

INTRODUCTION

PGs[2] serve as critical mediators in mammalian physiology, affecting a variety of functions, including blood vessel tone, platelet aggregation, and immune responses (1). In addition to normal processes, however, PGs have also been implicated in cancer development, with a number of tumor types found to produce more PGs than the normal tissues from which they arise (2–5). The rate-limiting enzyme in the synthesis of PGs from arachidonic acid is COX, which exists in two isoforms: COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and mediates the synthesis of PGs required for normal physiological function (6). In contrast, COX-2 is typically not expressed or is expressed at relatively low levels but can be induced by an assortment of agents, including proinflammatory stimuli, mitogens, and/or hormones, depending on the tissue (6). In addition, recent studies have shown COX-2 expression to be up-regulated in a number of human tumor types, including colon, pancreatic, prostate, gastric, and head and neck cancers (7–11). There is also considerable evidence available suggesting a causal relationship between COX-2 overexpression and colorectal tumorigenesis (2, 12). Thus, the preferential expression of COX-2 in tumors *versus* normal tissues suggests that this enzyme may provide a potential target for cancer therapy.

Although inhibitors of COX-1 and COX-2 have received considerable attention in cancer chemoprevention studies (13–16), there are also data from several experimental models indicating that these agents can also have antitumor activity. The NSAIDs sulindac, indomethacin, and ibuprofen, which are nonselective inhibitors of both COX-1 and COX-2, have been reported to reduce the proliferation rate of tumor cell lines grown *in vitro* and *in vivo* (17–19). In addition, there is also evidence suggesting that NSAIDs can act as radiosensitizing agents. Milas *et al.* (20) and Furuta *et al.* (21) have shown that indomethacin enhances the antitumor efficacy of ionizing radiation against PG-producing, transplanted murine sarcomas. More recently, ibuprofen has been shown to enhance the radiosensitivity of human prostate carcinoma cells grown *in vitro* and as xenografts in immunodeficient mice (17, 22). However, because standard NSAIDs are nonselective in their inhibition, affecting both COX-1 and COX-2, treatment with these agents is limited by normal tissue toxicity. Of particular sensitivity is the gastrointestinal tract, which is especially dependent on COX-1 for normal function (23, 24). Therefore, because it is COX-2 that is overexpressed in many tumor types, the recently developed selective COX-2 inhibitors may be more amenable

[2] The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; FCM, flow cytometry; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated nick end labeling; SF, surviving fraction; NSAID, nonsteroidal anti-inflammatory drug; CNS, central nervous system.

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for use in antineoplastic therapy both alone and in combination with radiation. In support of this concept, we recently reported that SC-236, a selective COX-2 inhibitor, caused a dramatic enhancement of the *in vivo* radiation response of NFSA (25) and FSA murine sarcomas (26). In contrast, the inhibitor had no influence on normal tissue damage by radiation.

The mechanism responsible for the enhanced tumor radioresponse induced by the COX-2 inhibitor has not been defined. Data from a murine model indicate that SC-236 can have an antiangiogenic effect (25). However, because PGs have been shown to have radioprotective properties (27), it is also possible that the inhibition of COX-2 may influence the intrinsic radiosensitivity of tumor cells. To investigate the effects of SC-236 on the intrinsic radiosensitivity of human tumor cells, we have performed a series of studies using the glioblastoma cell line U251. In addition to determining the effects of this COX-2 inhibitor on the intrinsic radioresponse of tumor cells, this cell line was also used as a means of exploring the potential for applying such compounds in combination with radiation for brain tumor treatment. Although treatment for a wide variety of human tumors could potentially improve with the use of a relatively nontoxic, selective radiosensitizer, particular benefits may be obtained for gliomas. Radiation is a major treatment modality for brain tumors in general, and despite the use of all available therapy, the median survival time for patients with glioblastoma multiforme, which accounts for the majority of human brain tumors, is only 1–2 years (28). With respect to COX-2, there is a low level of constitutive expression in the CNS that seems to be localized in specific neuronal populations and not in glial cells (29). Although COX-2 expression in gliomas has not yet been systematically compared with its expression in their normal glial counterparts, brain tumors have been reported to contain elevated levels of PGs (5). We now report on the *in vitro* and *in vivo* effects of the selective COX-2 inhibitor SC-236 alone and in combination with radiation on U251 glioblastoma cells.

MATERIALS AND METHODS

Cell Culture. The human glioblastoma cell line U251 was obtained from Dr. T. Mikkelsen (Henry Ford Hospital, Detroit, MI). Cells were grown in MEM (Life Technologies, Grand Island, NY) supplemented with gentamicin (10 μg/ml), sodium pyruvate (1 mM), and 10% fetal bovine serum. Cells were grown as monolayers in 75-cm^2 flasks and were maintained in a humidified 5% CO_2/95% air atmosphere at 37°C.

Cell Survival Analysis. Cells in monolayer culture were exposed to 50 μM SC-236 (Searle, Skokie, IL) or vehicle (100% ethanol) for 24 or 48 h and irradiated with graded doses of γ-rays using a ^{137}Cs source (3.7 Gy/min). Cells that remained attached were removed from the culture flask by exposure to 0.05% trypsin/1 mM EDTA solution and replated in specified numbers into 60-mm dishes in drug-free media for determination of colony-forming ability. After 10 days of incubation, the contents of the dishes were stained with 0.5% crystal violet in absolute methanol, and colonies with >50 cells were determined. Radiation survival curves were constructed after normalization for the cytotoxicity induced by the drug alone. Survival curves were generated by combining data from three to four independent experiments and fitting the average survival levels by least squares regression using the linear quadratic model (30).

Cell Cycle Phase Analysis. Evaluation of cell cycle phase distribution was performed at the FCM core facility at The University of Texas M. D. Anderson Cancer Center. The treatment protocols were essentially the same as in the clonogenic survival experiments. All of the cultures were subconfluent at the time of collection. Cultures were collected for fixation with ice-cold 70% ethanol, stained with propidium iodide, and analyzed using FCM as described previously (31). Attached cells (those remaining adhered to the dish) and floating cells (those having detached from the monolayer) were stained separately in some experiments.

Apoptosis Assay. The TUNEL assay (APO-DIRECT, PharMingen, San Diego, CA) was performed according to the manufacturer's instructions and analyzed basically as described by Gonczyca *et al.* (32). Briefly, fixed cells were washed in PBS, suspended in 50.75 μl of TdT buffer with 0.75 μl of TdT enzyme and 8.0 μl of FITC-dUTP. After overnight incubation at room temperature, cells were rinsed in buffer twice and resuspended in 1 ml of propidium iodide/RNase A solution. After incubating the cells in the dark for 30 min at room temperature, the specimens were analyzed using FCM.

***In Vivo* Growth Delay Assay.** Tumors were generated by injecting 5×10^6 U251 cells s.c. into the right thighs of 4-month-old *nu/nu* NCR mice. When tumors grew to 5 mm in diameter, the mice were given SC-236 or vehicle in drinking water for 10 consecutive days. SC-236 was dissolved in a stock solution of 5% Tween 20 and 95% polyethylene glycol and diluted in distilled water to achieve a final SC-236 concentration of 0.045 mg/ml. The mice drank approximately 4 ml/day, which corresponded to an SC-236 dose of 6 mg/kg body weight per day. Control animals received drinking water with vehicle only. The water bottles were changed every 3 days. When tumors grew to 7 mm in diameter, they were exposed to a 10-Gy single dose of γ-radiation using a ^{60}Co irradiator. To obtain tumor growth curves, three mutually orthogonal diameters of tumors were measured daily with a vernier caliper, and the mean values were calculated. Regrowth of tumors was followed until tumor diameter reached approximately 14 mm. Tumor growth delay was expressed as the time in days for tumors in the treated groups to grow from 7 to 12 mm in diameter minus the time in days for tumors in the control group to reach the same size. Four experimental arms were studied: (*a*) animals receiving vehicle only (controls); (*b*) animals receiving SC-236 alone; (*c*) animals receiving a single 10-Gy dose of radiation alone; and (*d*) animals receiving a combination of SC-236 and single dose of 10-Gy irradiation. The groups consisted of 7 to 10 mice each.

Northern Blotting. Total RNA was isolated from cell monolayers using RNAzol (CINNA/Biotex Laboratories, Friendswood, TX) according to the manufacturer's instructions. RNA samples (30 μg/lane) were separated on a 1% agarose-formaldehyde gel and blotted onto Zeta-Probe GT genomic membranes (Bio-Rad Laboratories, Hercules, CA). A human cDNA COX-2 probe was obtained from Oxford Biomedical Research (Oxford, MI). Membranes were hybridized with ^{32}P-cDNA probes by random primer extension (Boehringer Mannheim, Indianapolis, IN) in Hybrizol (Oncor Inc., Gaithersburg,

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MD) at 42°C and washed twice in 2× SSPE (300 mM sodium chloride, 200 mM sodium phosphate, and 200 mM EDTA) and 0.1% SDS at room temperature, once in 0.2% SSPE and 0.1% SDS at room temperature, and once in 0.1× SSPE at 42°C. Visualization and quantification of mRNA levels were performed using a Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA).

Western Blotting. Cells from *in vitro* samples were washed twice with ice-cold PBS, scraped in ice-cold PBS, and collected as whole cell lysates, which were centrifuged at 14,000 rpm for 10 min at 4°C. For *in vivo* tumor samples, fresh nonnecrotic tumor tissue was homogenized using a glass-on-glass tissue homogenizer, thawed in ice-cold lysis buffer [50 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 5 μg/ml benzamidine, and 1% NP40], and incubated on ice for 30 min. The protein concentration of the supernatant in each sample was determined using the Bio-Rad Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. A total of 60 μg of the supernatant was resolved by SDS-PAGE. After electrophoresis, the proteins were electroblotted onto an Immobilon-P membrane (Amersham Corp., Arlington Heights, IL) and blocked at room temperature for 5 h using TBS-T (Tris-buffered saline and 5% nonfat milk in 0.1% Tween 20). The membrane was probed with COX-2 polyclonal antibody (Oxford Biomedical Research) in the blocking mixture overnight at 4°C. The membrane was washed in TBS-T and incubated for 40 min with antirabbit IgG horseradish peroxidase-conjugated secondary antibody at a 1:2000 dilution in the blocking mixture. The membrane was washed with TBS-T and probed with ECL Plus (Amersham Corp.). Visualization and quantification were performed using the Storm 860-blue chemifluorescence scanner (Molecular Dynamics).

Statistical Methods. Statistical analysis was performed using Statistica 5.1 software (StatSoft, Tulsa, OK). Comparisons of means were carried out by Student's *t* test; differences with a value of $P < 0.05$ were considered statistically significant.

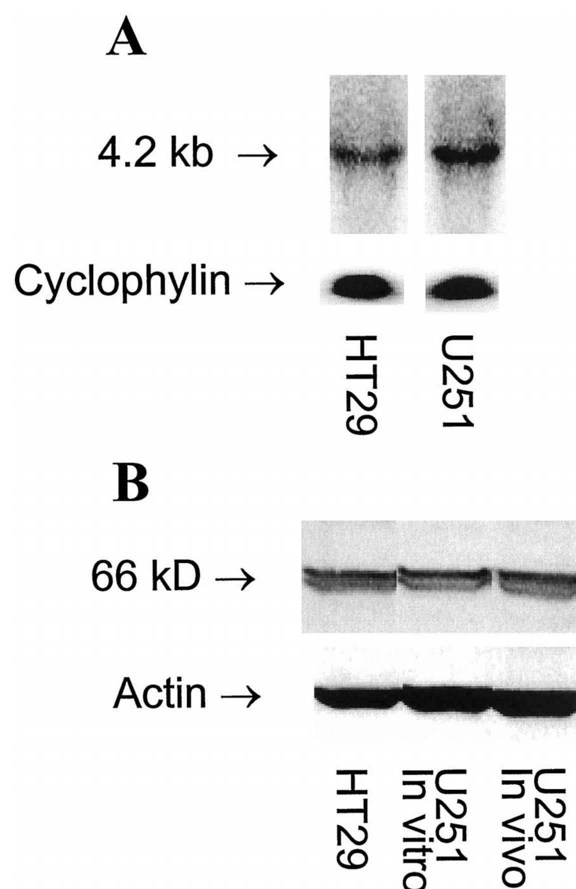

Fig. 1 Expression of COX-2 mRNA and protein in U251 cells. Representative Northern and Western blots are shown and, for each, the human colon cancer cell line HT29 is used as a positive control. *A*, total RNA was extracted from the designated cell line and subjected to Northern analysis. Cyclophilin was used as a control for gel loading. *B*, Western analysis of COX-2 protein levels in cell lines and U251 xenografts. Actin was used as a control for gel loading.

RESULTS

COX-2 mRNA and Protein Expression. The presence of COX-2 mRNA was evaluated by Northern blot analysis. The human colon cancer cell line HT29, which constitutively expresses COX-2, was used as a positive control (33, 34). In Fig. 1*A*, the constitutive expression of COX-2 is illustrated by a band at 4.2 kb, which is consistent with the size of COX-2 mRNA (6). Western blot analysis of cell lysates using an antihuman COX-2 polyclonal antibody revealed a specific COX-2 protein expression in the form of a 66-kDa band (Fig. 1*B*). It has been reported previously that four potential *N*-glycosylation sites are present in COX-2, which are variably occupied, depending on cell type (35). Thus, the difference in the molecular weight of the maximum glycosylated form of COX-2 (M_r 72,000) and M_r 66,000 COX-2 detected in U251 cells is consistent with differences in the used glycosylation sites, as reported by others (33, 35). Together, the Northern and Western blot analyses indicate that there is constitutive COX-2 expression in the U251 cell line. Exposure of U251 cells *in vitro* to SC-236 had no effect on COX-2 protein levels as detected by Western analysis (data not shown).

Effect of SC-236 Alone. As an initial characterization of the response of U251 cells to SC-236, a clonogenic cell survival assay was performed on monolayer cultures exposed to 50 μM SC-236 for 1 and 2 days (Fig. 2*A*). Although the SF was reduced to 0.1 after 1 day of drug incubation, 2 days of SC-236 treatment resulted in significantly less cell killing. This was an unexpected result; typically, an increase in the length of drug exposure leads to an increase in the amount of cell killing. However, it was noted that compared with 1 day of incubation, after 2 days of SC-236 exposure, a significant number of cells had detached from the monolayer and were floating in the medium. The colony-forming efficiency assay used in these studies evaluates the survival of only those cells attached to the flask. Therefore, to determine whether the observed differences in the SF at 1 and 2 days of SC-236 exposure can be accounted for by cell detachment, cells floating in the media were quantified at each time point. As shown in Fig. 2*B*, only a small increase in the amount of floating cells was detected after 1 day of SC-236 exposure.

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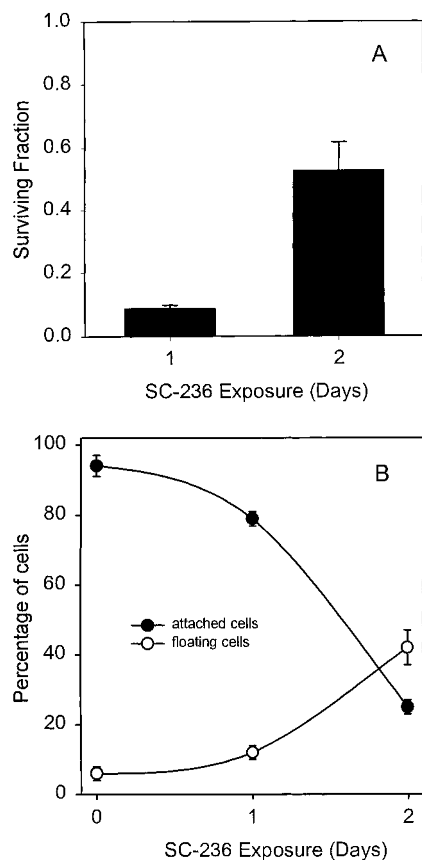

Fig. 2 Effect of SC-236 alone on U251 cells *in vitro*. *A*, cultures were exposed to SC-236 (50 μM) for 1 or 2 days. Cells remaining attached to the flask were then removed and plated at specified numbers, and colony-forming efficiency was determined. Values shown represent the means ± SE for 3–4 independent experiments. *B*, percentage of attached *versus* floating U251 cells after treatment with SC-236 for 1 and 2 days. Values shown, the means ± SE for three to four independent experiments.

However, after 2 days of drug incubation, a significant increase occurred in the proportion of floating cells, which corresponded to a decrease in the proportion of attached cells.

The frequency of apoptosis in monolayer cultures is often reflected by the number of cells that have detached from the monolayer floating in the medium. Elder *et al.* (34) reported that treatment of colorectal cell lines with a COX-2-selective NSAID increased the proportion of floating cells, which were found to be apoptotic. To determine whether the floating cells appearing after SC-236 treatment were apoptotic, the TUNEL assay was performed on the attached and floating cell populations. After day 1 of SC-236 treatment, no apoptosis was detected in either population (data not shown). After 2 days (Fig. 3), only 1.2% of the attached cells were apoptotic compared with 82.6% of the floating cells. Thus, the difference in colony-forming efficiency obtained at 1 and 2 days of SC-236 treatment is explained by the characteristics of the cell-survival assay used and the mode of cell death induced by SC-236, *i.e.,* SC-236 kills U251 cells through the induction of apoptosis, which results in cell detachment from the monolayer. This process takes >24 h. After only 1 day of drug incubation, all of the cells, including those destined to die via apoptosis, remain attached to the flask in monolayer and are analyzed by the colony-forming efficiency assay, which results in a low SF. However, after 2 days, the floating cells, *i.e.,* cells that died because of apoptosis, are eliminated from the assay during the trypsinization process, and only the nonapoptotic attached cells are analyzed.

Effect of SC-236 on Cell Radiosensitivity. To determine the influence of SC-236 on the sensitivity of U251 cells to ionizing radiation, cells in monolayer culture were exposed to 50 μM SC-236 for 24 or 48 h and irradiated with graded doses of γ-rays. The cells were then assayed for colony-forming efficiency; the survival curves generated were corrected for cell killing induced by the drug alone. As shown in Fig. 4, SC-236 treatment for 1 day had no significant effect on the radiosensitivity of U251 cells compared with the untreated control cells. Treatment of U251 cells with SC-236 for 2 days, however, resulted in a significant increase in the level of radiation-induced cell death. The dose enhancement factor at a SF of 0.1 was 1.4, and the SF at 2 Gy was reduced from 0.61 to 0.31. The failure to observe radiosensitization after 1 day of SC-236 exposure may involve the significant level of cell death induced by the drug alone at this time point. As shown in Fig. 2*A*, SC-236 alone reduces survival by 90%; it is unlikely that radiosensitization could be detected in the remaining 10% of the population. However, at 2 days, the dead (apoptotic) cells were eliminated, and radiosensitization was clearly detectable in the remaining attached cells.

The accumulation of cells in the radiosensitive G_2-M phase of the cell cycle was reported as a possible mechanism by which NSAIDs enhance murine tumor radioresponse *in vivo* (21). To determine whether this mechanism is involved in SC-236-induced radiosensitization of U251 cells *in vitro*, the cell cycle phase distribution of U251 cells was analyzed after 2 days of treatment with SC-236 alone, which corresponds to the time of radiosensitization. As illustrated by DNA histograms in Fig. 5, at 2 days after drug treatment, only minor changes in U251 cell cycle phase distribution were observed. The percentage of cells in the G_1, S, and G_2-M cell cycle phases for control and SC-236-treated cells were 75, 11, 14, and 72, 20, 8, respectively. Thus, an accumulation of cells in the radiosensitive G_2-M phase of the cell cycle does not seem to account for the radiosensitization. Another possible mechanism for SC-236-mediated radiosensitization is the disruption of a cell cycle delay typically induced by radiation. As shown in Table 1, radiation alone induces a G_2-M block in U251 cells; SC-236 does not affect this cell cycle delay. At 3 and 4 days after SC-236 alone, the U251 cell cycle phase distribution was essentially the same as in Fig. 5 for 2 days of treatment (data not shown). To determine whether the SC-236-mediated radiosensitization of U251 cells involves an increase in the propensity of these cells to undergo apoptosis, flow cytometric analysis of TUNEL-stained cells was performed. For SC-236 treatment alone, after the apoptosis that occurred after 2 days (Fig. 3), no additional apoptosis was detected (data not shown). Furthermore, no increase in the percentage of apoptotic cells after the combination of SC-236 and irradiation was detected (Table 1). Thus, the data in Fig. 4 and Table 1 suggest that SC-236-mediated radiosensitization is

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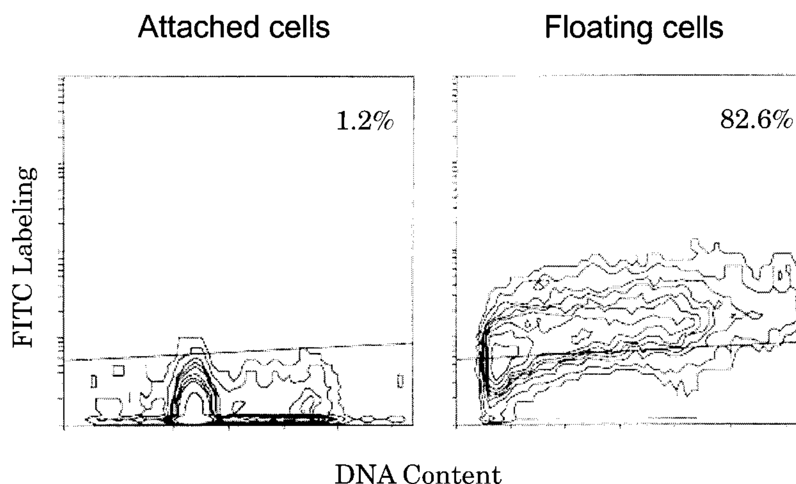

Fig. 3 Induction of apoptosis by SC-236. U251 cell cultures were exposed to SC-236 (50 μM) for 2 days; floating and attached cells were then collected separately. Apoptosis was determined by FCM analysis of TUNEL-stained cells. The histograms shown are representative of two independent experiments.

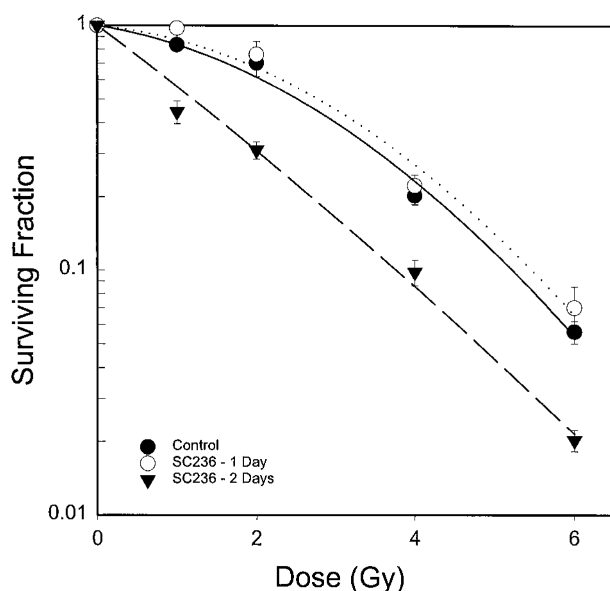

Fig. 4 Effect of SC-236 on *in vitro* radiosensitivity of U251 cells. Cultures were treated with SC-236 (50 μM) for 1 or 2 days before irradiation. After irradiation, attached cells were removed from the flask by trypsinization and plated in specified numbers. Colony-forming efficiency was determined 10 days later, and survival curves were constructed after normalizing for the cytotoxicity induced by SC-236 alone. Values shown, the means ± SE for three to four independent experiments.

the result of an enhancement of radiation-induced clonogenic cell death.

Effect of SC-236 on Tumor Radioresponse *in Vivo*. To determine whether the *in vitro* effects of SC-236 can be extended to an *in vivo* model, U251 cells were grown as tumor xenografts in the hind leg of nude mice. Western blot analysis of control tumors revealed that the expression of COX-2 *in vivo* is similar to its expression *in vitro* (Fig. 1*B*). The effects of SC-236 on the *in vivo* radioresponse of this human tumor cell line were evaluated using a protocol found previously to enhance the radioresponse of a murine tumor grown in syngenic hosts (25). Animals bearing 5-mm-diameter tumors were given SC-236 in drinking water (0.045 mg/ml) for 10 consecutive days. A single dose of 10 Gy was given when these tumors grew to 7 mm in diameter, which occurred 4–6 days later for both control and drug-treated groups. Figure 6*A* shows that SC-236 treatment alone inhibited tumor growth significantly; the time required for tumors to grow from 5 to 12 mm in diameter was increased from 8.9 ± 0.9 to 13.6 ± 0.7 days ($P < 0.01$). To assess whether SC-236 enhances tumor radioresponse, tumor-bearing mice were treated with SC-236 alone, radiation alone, or the combination of the two, and tumor growth was measured. As shown in Fig. 6*B*, SC-236 greatly increased radiation-induced growth delay. The absolute growth delays (the time in days for tumors to grow from 7 to 12 mm in treated mice minus the time in days for tumors to reach the same size in untreated mice) were 4.3 for SC-236 treatment only, 9.9 for radiation only, and 25.4 for the combination. Thus, the growth delay after the combined treatment was more than the sum of growth delays caused by radiation alone or SC-236 alone. The dose enhancement factor, obtained by dividing normalized tumor growth delay in mice treated with SC-236 plus radiation with the absolute growth delay in mice treated with radiation alone, was 2.13. Normalized tumor growth delay is defined as the time in days for tumors to grow from 7 to 12 mm in mice treated with the combination of SC-236 and radiation minus the time in days for tumors to grow from 7 to 12 mm in mice treated with SC-236 alone. Thus, SC-236 alone slows tumor growth and enhances the effect of ionizing radiation, with results similar to the results obtained *in vitro*. No observable toxicity was detected in any of the mice, which is consistent with the preferential enhancement of tumor radioresponse compared with that of normal tissue, as recently reported by Kishi *et al.* (26).

DISCUSSION

The median survival time for the majority of patients with brain tumors remains poor, despite the use of all available treatments. Thus, there is a critical need to identify novel therapeutic targets for single and combined modality approaches to brain tumor treatment. Recent studies have suggested that

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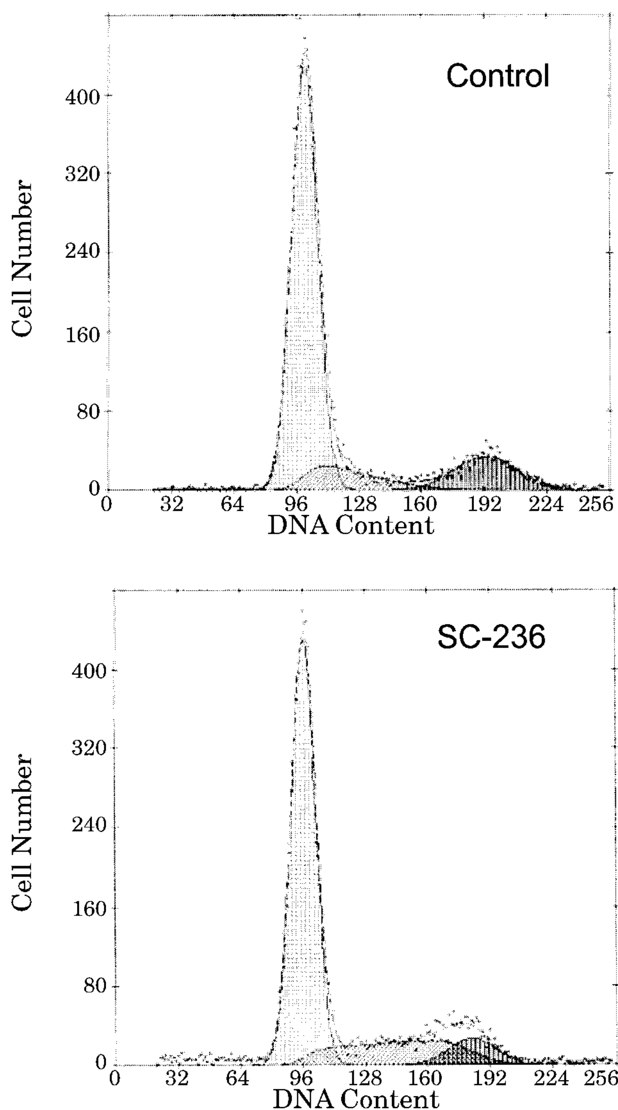

Fig. 5 Effect of SC-236 on U251 cell cycle phase distribution. Cultures were exposed to SC-236 (50 μM) for 2 days before collection and FCM analysis of propidium iodide-stained cells. The histograms shown are representative of two independent experiments.

Table 1 Effect of SC-236 on radiation-induced cell cycle phase redistribution and apoptosis

Cultures were treated with SC-236 (50 μM) for 2 days and then irradiated with 6 Gy. The medium was then replaced in each culture, and cell cycle phase distribution and the percentages of apoptotic cells were determined at 1 and 2 days after irradiation. Values shown are representative of two independent experiments.

	Cell cycle distribution			
Treatment	G_1 (%)	S (%)	G_2-M (%)	Apoptotic cells (%)
Control	75.2	10.7	14.1	0.4
6 Gy/1 day	47.3	3.9	48.8	0.01
6 Gy/2 days	41.8	16.5	41.7	0
SC-236 + 6 Gy/1 day	24.9	11.2	63.9	0.4
SC-236 + 6 Gy/2 days	47.6	8.8	43.6	0.04

COX-2 may serve in such a role. Within the CNS, there is a low constitutive level of COX-2 expression in specific neuronal populations but not in the considerably more prevalent glial cells. Although its function in the CNS has not been defined, COX-2 is induced after excitotoxic injury and ischemia and has been associated with the expression of these two types of injury in experimental models (36–40). The expression of COX-2 is also elevated in neurodegenerative conditions such as Alzheimer‘s disease, and several studies have demonstrated the protective action of NSAIDs against cognitive decline in patients with Alzheimer's disease (41–43). Thus, COX-2 induction in the CNS has been linked to neuronal loss. Nonspecific NSAIDs have not been reported to result in any CNS toxicity, in contrast to their toxic effects on the gastrointestinal tract (43). Therefore, preferential expression of COX-2 in gliomas may provide a potential target for brain tumor therapy.

The data presented here show that the glioma cell line U251 expresses COX-2 at a level similar to that in the human colon cancer cell line HT29. Furthermore, exposure of U251 cells to SC-236 results in a significant reduction in cell survival, which can be attributed to the induction of apoptosis. This is similar to HT29 cells in which apoptosis was detected after exposure to the COX-2 inhibitor NS-398 (34). However, in addition to reducing cell survival by approximately 90%, SC-236 enhanced the radiosensitivity of the remaining surviving cells. The antitumor and radiosensitizing effects of SC-236 were also detected in U251 cells grown as xenografts in nude mice. To our knowledge, this is the first demonstration of a selective COX-2 inhibitor affecting the intrinsic radiosensitivity of tumor cells. We recently reported (25) that SC-236 delivered in drinking water enhanced the *in vivo* radioresponse of a murine sarcoma designated NFSA, as determined by tumor growth delay and the dose yielding 50% tumor cure. In that study, the COX-2 inhibitor was shown to reduce NFSA neovascularization, which correlated with the radiosensitizing effect. The involvement of antiangiogenesis is a possibility, based on the increasing evidence demonstrating that the therapeutic effect of the combination of radiation and antiangiogenic compounds, such as angiostatin or TNP-470, is greater than additive (44, 45). Whether SC-236 enhanced the intrinsic radiosensitivity of the murine NFSA cells or whether SC-236 inhibited neovascularization of U251 cells *in vivo* remains to be determined. However, these studies illustrate two processes by which COX-2 inhibition can enhance the *in vivo* response of tumors to radiation.

The cellular and biochemical processes responsible for the SC-236-mediated radiosensitization remain to be determined. Furuta *et al.* (21) showed that indomethacin, a nonspecific NSAID, caused accumulation of cells in the G_2-M phase of the cell cycle, which is generally considered to be the most sensitive to ionizing radiation. As illustrated by DNA histograms (Fig. 5), no increase in the percentage of U251 in G_2-M was observed 2 days after drug treatment, which corresponds to the time of radiosensitization. Another possible mechanism would be a disruption in cell cycle delay typically induced by radiation. As shown in Table 1, radiation alone induces a G_2-M block in U251 cells, but SC-236 does not affect this cell cycle delay. Although

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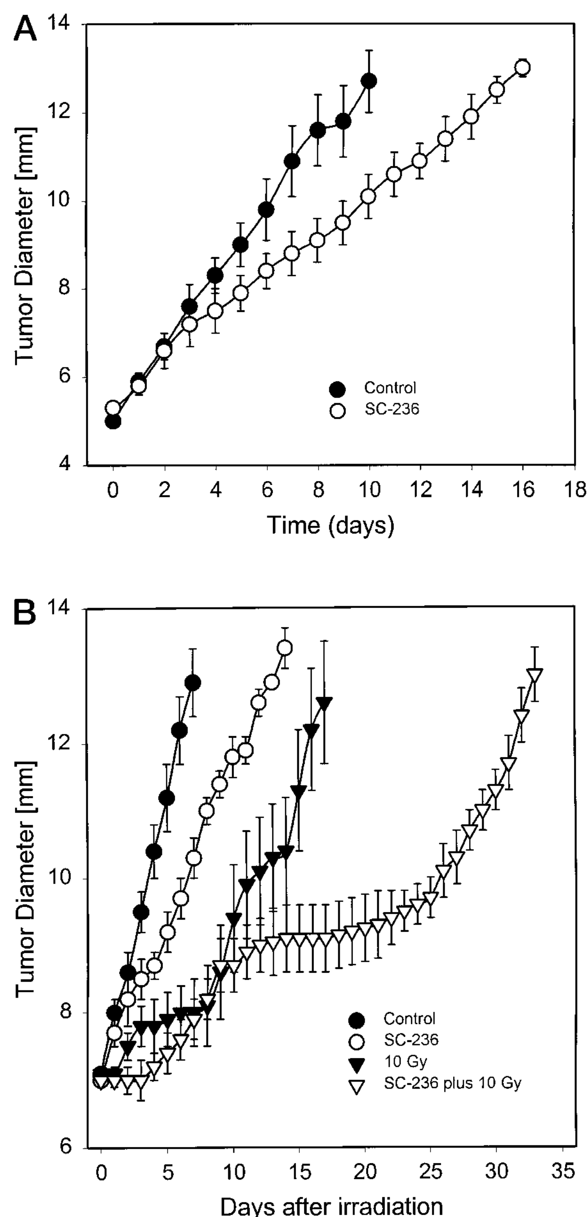

Fig. 6 Effect of SC-236 on the *in vivo* radioresponse of U251 cells. U251 cells were injected into the hind leg of *nu/nu* mice and grown as tumor xenografts. SC-236 treatment (0.045 mg/ml of drinking water, 6 mg/kg of body weight per day) was initiated when tumors were 5 mm in diameter and was continued for 10 days. Local radiation (10 Gy) was delivered when tumors were 7 mm in diameter, which occurred 4–6 days later for both treated and control mice. *A*, effect of SC-236 alone on tumor growth; ●, vehicle control mice; ○, treated mice. *B*, effect of the combination of SC-236 treatment and local irradiation on tumor growth: vehicle control (●), SC-236 (○), 10 Gy single dose (▼), SC-236 plus 10-Gy single-dose irradiation (▽). Values shown, the means ± SE of 7–10 mice.

induction of G_2-M arrest after treatment with NSAIDs has been reported for colon cancer cell lines (46), the data presented here suggest that cell cycle modification is not involved in the SC-236-induced radiosensitization of U251 cells.

An additional process that may account for the effects of SC-236 is the shifting of the mode of radiation-induced cell death from clonogenic to apoptotic, which has received considerable attention as a means of radiosensitization. Indeed, Elder *et al.* (34) recently reported an increase in the number of detached cells from cultured human colon carcinoma cell lines in response to NS-398, a COX-2 selective inhibitor, which was attributable almost entirely to the induction of apoptosis. To determine whether the SC-236-mediated radiosensitization of U251 cells involves an induction of apoptosis, TUNEL staining was performed after combined treatment. In contrast with the apoptosis that occurred after 2 days of treatment with SC-236 alone, no increase in the percentage of apoptotic cells after the combination of SC-236 with irradiation was detected, which suggests that SC-236-mediated radiosensitization is the result of an enhancement of radiation-induced clonogenic cell death.

In summary, our findings demonstrate that the selective COX-2 inhibitor SC-236 is cytotoxic to U251 glioma cells *in vitro* because of induction of apoptosis and that it enhances the radiosensitivity of the surviving cells. Furthermore, these *in vitro* results correlated with the effects of SC-236 alone and in combination with radiation on U251 xenografts. Thus, the selective inhibition of COX-2 in combination with radiation may have potential as a treatment for human brain tumors.

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