Synergy between Celecoxib and Radiotherapy Results from Inhibition of Cyclooxygenase-2-Derived Prostaglandin E$_2$, a Survival Factor for Tumor and Associated Vasculature

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ABSTRACT

Previous work has demonstrated that selective cyclooxygenase-2 (COX-2) inhibitors can act synergistically with radiotherapy to improve tumor debulking and control in preclinical models. The underlying mechanism of this remarkable activity has not yet been determined. Here, we report that radiation can elevate intratumoral levels of COX-2 protein and its products, particularly prostaglandin E$_2$ (PGE$_2$). Furthermore, inhibition of COX-2 activity or neutralization of PGE$_2$ activity enhances radiotherapy even in tumors where COX-2 expression is restricted to the tumor neovasculature. Direct assessment of vascular function by direct contrast enhancement–magnetic resonance imaging showed that the combination of radiation and celecoxib lead to enhanced vascular permeability. These observations suggest that an important mechanism of celecoxib-induced radiosensitization involves inhibition of COX-2-derived PGE$_2$, thus removing a survival factor for the tumor and its vasculature.

INTRODUCTION

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as indomethacin have been reported to potentiate the lethal effect of radiation on tumor cells and conversely to protect irradiated immune cells in vitro and in vivo (1) at doses that have been shown to inhibit prostaglandin production (2). NSAIDs are known to interfere with the ability of the cyclooxygenase-1 and -2 (COX-1 and COX-2) to convert arachidonic acid to prostaglandins. However, the in vivo inhibition of COX-1 by these compounds is thought to have undesirable side effects on proliferating tissues such as gastrointestinal mucosa (reviewed in Ref. 3). The recent observation that disruption of COX-1 alleles renders the resultant transgenic mice hypersensitive to radiation damage and subsequent loss of intestinal crypt cells (4) lends support to the notion that inhibition of COX-1 activity can be detrimental to physiological mucosal maintenance after radiation damage. The discoveries of an inducible isoform of COX (COX-2) and compounds that selectively inhibit this isoform provide the opportunity to differentiate between physiological prostaglandins; i.e., those associated with maintenance of gastric mucosa versus those associated with pathological conditions such as inflammation and cancer. COX-2 is reported to be present or induced in inflammatory conditions such as rheumatoid arthritis and osteoarthritis and in cancers. A recent report suggests that elevated COX-2 expression correlates with reduced patient survival after radiation therapy (5). These observations raise the possibility that COX-2 may protect tumor cells from damage by generating prostaglandins as tumor survival factors. This possibility is supported by the report that COX-2 protein can be further induced following ionizing radiation (IR) in tumor cells in vitro (6). We tested this hypothesis in several tumor models using selective COX-1 and COX-2 inhibitors and a neutralizing antibody to prostaglandin E$_2$ (PGE$_2$).

COX-2 is expressed in neoplastic epithelial cells in a wide variety of human tumor types (7–9). It has also been shown that several types of epithelial cancers produce high levels of prostaglandins, particularly PGE$_2$ (10–12). In addition to neoplastic epithelia, COX-2 is highly expressed in the microvasculature in many human tumors (7–9). This suggests that COX-2-derived PGE$_2$ may support tumor growth through angiogenic endothelial cell growth and blood vessel formation (13, 14). We have shown previously that selective COX-2 inhibitors limit fibroblast growth factor-mediated angiogenesis in a rat corneal micropocket assay and that ocular levels of prostaglandins, including PGE$_2$, are reduced in the process (15, 16). We have also reported previously that the COX-2 inhibitor celecoxib inhibits tumor growth in a number of murine tumor models regardless of whether the tumor cells themselves express COX-2 (9, 15, 16). We hypothesized that because COX-2 is found in the stroma of all of these models, the primary mechanism of action of celecoxib within these models is antiangiogenesis (9, 16, 17). This hypothesis is supported by Williams et al. (18), who showed that host COX-2 is important for tumor neovascularization and subsequent tumor growth. Numerous researchers have reported increased levels of various eicosanoids, including prostaglandins, in response to IR (18–28), particularly in vitro. It has also been observed that prostaglandins can play a role in cell survival after IR. The addition of prostaglandins or their analogues has been shown to be radioprotective in a number of cell types (29–36) and in mice (37). In contrast, use of the COX-2-selective inhibitor celecoxib has been shown to have radiosensitizing and chemosensitizing activities in vitro (38), although it should be noted that the concentrations of compound used exceed those necessary to inhibit COX-2-mediated prostaglandin synthesis. The mechanism(s) of the radioprotection by prostaglandins (36) or the radiosensitization by celecoxib observed in vitro is unknown. In murine tumor models, several COX-2 inhibitors, including celecoxib when used at physiological doses, have shown clear synergistic responses when combined with radiotherapy (39–44).

To help elucidate the in vivo mechanism whereby COX-2 inhibitors enhance radiotherapy, we used three murine tumor models. One model expresses COX-2 only in the tumor stroma, and two models express COX-2 within the stroma and the tumor epithelia. A human colon carcinoma model, HT29, implanted in the footpad of nude mice expresses COX-2 only in the tumor stroma, particularly the neovasculature (45). This model provides us the opportunity to observe the combined effect of COX-2 inhibition and radiation on tumor neovascularization without interference from COX-2-derived prostaglandins from the tumor cells themselves. Two other murine models, human head and neck squamous cell carcinoma (1483 HNSCC) and murine colon cancer [colon-26 (Col26)], implanted into the footpads of mice express COX-2 in tumor epithelial cells and tumor-associated stroma, resembling COX-2 expression patterns found in human cancer. These models, derived from vastly different epithelia, allowed us to assess the impact of inducible versus constitutive COX-2 and PGE$_2$ levels on
tumor debulking and regrowth after IR. 1483 HNSCC expresses COX-2 in pockets of tumor epithelial cells and tumor-associated stroma (45). We have observed that levels of COX-2 and PGE2 are acutely elevated after IR in these tumors. Here, we also report that inhibition or inactivation of the survival factor PGE2 by either celecoxib or a specific PGE2-neutralizing antibody enhances the antitumor activity of radiation, suggesting that COX-2-derived PGE2 may be a primary target for the radiosensitization observed with celecoxib. We used the murine Col26 model to evaluate, by contrast magnetic resonance imaging (MRI), the effects of COX-2 inhibition and radiation on tumor neovascular integrity and function. These tumors express very high levels of COX-2 protein and PGE2, with intratumoral levels of 1.1–3.4 μg PGE2/g tissue. Using this model, it was observed that addition of celecoxib to radiotherapy causes increased permeability of the tumor neovasculature.

Taken together, these data suggest that celecoxib synergizes with radiotherapy by inhibiting COX-2-derived PGE2 production, which leads to greater antitumor efficacy by preventing the survival of the tumor neovasculature.

MATERIALS AND METHODS

Col26 Murine Carcinoma. Col26 cells (1 × 106) were suspended in 30% Matrigel-HBSS and injected in the right hind footpad of male BALB/c mice (n = 6–8 mice/group). Drug treatment began when tumors reached a mean of 0.48–0.56 ml. Tumors were irradiated with a single dose of 6 Gy on the day after the start of drug treatment. 1483 HNSCC. 1483 HNSCC cells (1 × 106) were suspended in 30% Matrigel-HBSS and injected in the right hind footpad of male athymic (nude) mice (n = 8 mice/group). Mouse anti-PGE2 monoclonal antibody (2B5) or isotype-matched IgG control antibody was administered at 10 mg/kg three times/week i.p. Antibody treatment and drug treatment started once tumors reached a mean of 0.36–0.39 ml. Tumors were irradiated with a single dose of 6 Gy on the day after the start of drug treatment.

HT-29 Human Colon Carcinoma. HT-29 cells (1 × 106) were suspended in 30% Matrigel-HBSS and injected in the right hind footpad of male athymic (nude) mice (n = 6 mice/group). Drug treatment started when tumors reached a mean of 0.3–0.35 ml (day −1). Tumors received fractionated radiation at 6 Gy/dose on days 0, 4, and 11.

Treatments. All drugs were orally gavaged twice daily in 0.1 ml of 0.5% methylcellulose and 0.025% Tween 20, e.g., celecoxib at 125, 12.5, or 2.5 mg/kg body weight was orally gavaged twice per day for a total dose of 250, 25, or 5 mg/kg/day. Radiation was administered in single doses of 6 Gy to the tumors using a dual-source 137Cs irradiator at a rate of 1 Gy/min. The mice were shielded using a Gamma-cell 40 Collimator centering the tumor in a 3-cm radiation field. Data are expressed as the mean tumor volume ± SE. Paw volumes were measured using a plethysmometer and final tumor volume calculated by subtraction of non-tumor-bearing left hind paw volume. Mouse body weights were monitored as an assessment of health. All animals used in the experiments described were treated humanely in accordance with Institutional Animal Care and Use Committee guidelines.

Prostaglandin Analysis. At the time of sacrifice, tumor tissue was snap frozen and stored at −80°C. Frozen tissues were ground in liquid nitrogen using a mortar and pestle, weighed, and extracted for prostaglandins in absolute methanol. The supernatants were dried under nitrogen, resuspended in ELISA buffer (Cayman Chemical, Ann Arbor, MI), and assayed for various prostanoids including PGE2, thromboxane B2, 6-keto-PGF1α, and PGF2α by ELISA.

MRI Analysis. Footpad tumors were imaged 1 day after radiation therapy. Each mouse was anesthetized with isoflurane during MRI preparation and examination, and a 26-g catheter was inserted into a tail vein to administer contrast agents. The tumor and catheter were secured and immobilized within a customized animal cradle. Rectal temperature, partial pressure of O2, and electrocardiogram signal were monitored throughout the MRI session. The initial uptake of each contrast agent was determined by measuring the initial MRI signal change within each tumor paw image pixel. Although the MRI signal change is an exponential function of contrast agent concentration, this relationship is approximately linear (R2 = 0.97) within the conditions of this protocol. The initial uptake of contrast agent represents a combination of tumor vascular permeability, surface area, and flow, which are physiological indicators of angiogenesis, vascular damage, or vascular repair.

The temporal profile for multiple slice direct contrast enhancement (DCE-MRI) data through the tumor was acquired using a T1-weighted two-dimensional gradient echo sequence on a Varian Inova system operating at 7.0 T equipped with a gradient insert (25 G/cm and 33 mm inner diameter). The following pulse sequence parameters were used: time of recovery, 60 ms; time of evolution, 3.0 ms; slice thickness, 1 mm; flip angle, 30 degrees; 256 × 128 matrix; and field of view, 30 × 30 mm. Five pre-injection images were acquired, 60 μmol/kg albumin-(gadolinium-diethylenetriaminepentaacetic acid)3- were then injected through a tail vein catheter, and 80 image sets were acquired for 41 min. Five additional pre-injection images were acquired, 30 μmol/kg gadolinium-diethylenetriaminepentaacetic acid were then injected, and 120 image sets were acquired for 15 min. The protocol was optimized for high-throughput examination of up to eight mice/day. Using these technical improvements, DCE-MRI data were acquired for more than 90% of the 14 h required to assess eight animals. Studies were staggered such that four animals of each treatment group (e.g., radiation versus combo; vehicle versus celecoxib) were scanned in the same day. Therefore, this protocol represented the highest possible throughput without compromising the integrity of the DCE-MRI results or the consistency of the tumor growth stage. Time-domain MRI data were processed using standard procedures with VNMR (Varian, Inc.). The temporal changes in DCE image contrast were analyzed for each pixel within the tumor for each contrast agent using customized routines with Matlab v6.1 (Mathworks, Inc.). Results were evaluated by measuring the MRI signal change normalized to the initial increase of contrast agent in the blood pool. Pixel selection criteria were based on initial area under the curve (IAUCs) relative to image noise variance.

Western Blot Analysis. Frozen tumor tissue was ground under liquid nitrogen and lysed in buffer containing 100 mM Tris-buffered saline (pH 8), 150 mM NaCl, 1% Tween 20, 50 mM diethyldithiocarbamate acid, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor mixture tablet with 1 mM EDTA (Boehringer Mannheim). Lysates were sonicated for 20 s on ice and centrifuged at 10,000 × g for 10 min. The supernatants were measured for protein using the Bradford Protein Assay (Bio-Rad). The protein (50 μg/lane) was loaded and separated by SDS-PAGE. SDS-PAGE was performed under reducing conditions on 10% polyacrylamide Tris/glycine gels. The resolved proteins were transferred onto Immobilon-P membrane (Millipore) using an XCell II Blot Module (Novex). The membrane was blocked and then probed with a rabbit polyclonal anti-COX-2 antiserum (PG-27; Oxford Bio-medical Research).

RESULTS

Synergy with Radiation Treatment Is Observed through Inhibition of COX-2-Derived PGE2 by Celecoxib, Enhancing Tumor Debulking. Celecoxib dose-dependently increased the effectiveness of radiotherapy. Large established Col26 tumors were treated with celecoxib (0, 5, 25, and 250 mg/kg/day) with or without a single dose of IR (6 Gy). IR initially slowed tumor growth, but tumors quickly recovered and continued to grow rapidly (Fig. 1A). The addition of celecoxib with radiation resulted in a dose-dependent synergistic enhancement in tumor growth inhibition. We define synergy when the percent tumor growth inhibition observed with the combination of agents is greater than the additive inhibitory effects of each substance alone. For example, on day 5 (Fig. 1A), average percent inhibition for celecoxib (250 mg/kg/day) treatment was 23% compared with vehicle treatment. Radiation treatment inhibited tumor growth by 56%. However, the combination of the two agents resulted in 98% tumor inhibition consistent with the synergy definition.

Celecoxib given at 5, 25, and 250 mg/kg/day resulted in 47, 91, and 112% inhibition compared with the radiation only group (Fig. 1A). At the highest dose tested, celecoxib combined with radiation produced tumor growth regression as defined as a lower average tumor size on day 9 post IR compared with average tumor size on the day that drug treatment started (day −1). On average (eight mice/group, n = 4

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experiments), combination of 250 mg/kg celecoxib/day with a single dose 6 Gy radiation increased the average time for large tumors to triple in size (0.5–1.5 ml volume) from 10.8 ± 0.48 days to 17.8 ± 0.85 days post treatment, an enhancement factor of 1.65 (17.8 days/10.8 days). Furthermore, treatment with celecoxib reduced intratumoral PGE2 levels from 1.4 to 0.3 μg/g (Fig. 1B).

To establish the possible contribution of COX-1-derived prostaglandins versus those generated by COX-2, a selective COX-1 inhibitor (SC-560), a nonselective NSAID (indomethacin), and a selective COX-2 inhibitor (celecoxib) were compared for their ability to enhance radiotherapy at doses that maximally inhibit their respective enzymes in this model. Celecoxib and indomethacin were slightly effective in tumor growth delay when given as single agents (Fig. 1C). More importantly, celecoxib and indomethacin showed enhancement in inhibiting tumor growth when combined with radiation. In contrast, the COX-1 inhibitor SC-560, at doses which fully blocked COX-1-
derived serum thromboxane (45), was ineffective at reducing tumor growth as a single agent and failed to increase the effectiveness of radiotherapy (Fig. 1C). Analysis of intratumoral prostaglandins demonstrated that on average, celecoxib (250 mg/kg/day) alone and in combination with radiation reduced PGE2 levels by 79 ± 5.35%, whereas indomethacin reduced the levels by nearly 100% alone and in combination. In contrast, although SC-560 inhibited intratumoral PGE2 levels by 40%, it did not enhance the efficacy of radiotherapy.

Although many reports have demonstrated that COX-2 and its products are present in many human tumors, the induction of COX-2 levels by radiation has only been shown in vitro. To address this possibility in vivo, the 1483 HNSCC was grown in nude mice, and COX-2 protein and prostaglandin levels were determined after 6 Gy of IR (Fig. 2). COX-2 is expressed in pockets of tumor cells and the tumor stroma (e.g., neovascularature), similar to the pattern reported in human head and neck cancer. COX-2 protein levels within tumors were induced compared with vehicle 48 h after IR as determined by Western blot analysis showed an increase in COX-2 protein (1.5-fold) at 48 h post IR. A, control COX-2 peptide; V, vehicle-treated animals; IR, irradiated tumors. B, PGE2 levels were also elevated by 6 Gy of IR to roughly 2-fold vehicle control at 24 and 48 h post treatment, assayed by ELISA (see “Materials and Methods”).

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Fig. 2. Radiation additionally induced COX-2 expression in head and neck 1483 HNSCC tumors and increased PGE2 levels. Large established 1483 HNSCC tumors grown in the footpad of nude mice were irradiated with 6 Gy of IR. A, Tumors that were harvested and analyzed for Western blot analysis showed an increase in COX-2 protein (>1.5-fold) at 48 h post IR. C, control COX-2 peptide; V, vehicle-treated animals; IR, irradiated tumors. B, PGE2 levels were also elevated by 6 Gy of IR to roughly 2-fold vehicle control at 24 and 48 h post treatment, assayed by ELISA (see “Materials and Methods”).

Fig. 3. Both celecoxib and a neutralizing antibody to PGE2 synergize with radiation in the 1483 HNSCC model. Tumors of 0.4 ml in volume were treated with celecoxib (250 mg/kg/day, twice per day) or a neutralizing antibody to PGE2 (2B5, 10 mg/kg three times/week, i.p.) with or without 6 Gy radiation. 2B5 and celecoxib demonstrated enhancement with radiation; however, it is of note that 50% of celecoxib + IR-treated animals (Cel + IR) had tumor cures with no tumor at the end of study (day 70). $n = 8$ /group.
COX-2 and its major metabolite, PGE2, in the response of tumors to radiotherapy. Celecoxib dose-dependently synergizes with radiation vascular damage through inhibition of PGE2 that serves as a survival factor in the context of radiotherapy. This could be a primary mechanism of the synergy observed between celecoxib and radiation. Results suggest that the radiosensitization effect observed in vivo with COX-2-selective inhibitors is a complex process involving tumor/stromal cellular interactions. Recently, Garcia-Barros et al. (49) used asmase-deficient and Bax-deficient mice to support the hypothesis that the radiosensitization effect observed in vivo with COX-2-selective inhibitors is a complex process involving tumor/stromal cellular interactions. Recently, Garcia-Barros et al. (49) used asmase-deficient and Bax-deficient mice to support this hypothesis by demonstrating that endothelial cell damage increases tumor cell response to radiation. Also, Wachtsberger et al. (50) have recently reviewed mechanisms of interaction produced during tumor response to IR when combined with antiangiogenesis or vascular targeting agents.

In this report, we use three in vivo mouse tumor models that express COX-2 at different levels (low, moderate/inducible, and high/constitutive) and in different areas of the tumor milieu (stromal only, stromal plus pockets of tumor epithelial cells, and throughout the tumor). We have demonstrated previously that celecoxib as a single agent is effective at reducing tumor growth by at least 70% in all three models when treatment is initiated at or before tumors reach 0.3 ml in volume (9, 45, 46). Tumor growth appears to be reliant on proangiogenesis or vascular targeting agents.

DISCUSSION

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hypothesis that the activity of celecoxib on these tumors is primarily by blocking angiogenic prostaglandin production (9, 16). We used these models to explore the target tissues and the mechanism(s) by which COX-2-derived prostaglandins may affect survival after radiation insult in large tumors that are refractory to single-agent therapies. We demonstrated that celecoxib dose-responsively enhanced radiotherapy in the Col26 model. Celecoxib and indomethacin demonstrated similar effects with radiation, whereas a selective COX-1 inhibitor did not. The enhancements observed with celecoxib or indomethacin (but not the COX-1 inhibitor SC-560) suggest that COX-2 inhibition is the common and most significant activity of both compounds with radiation. Interestingly, SC-560, while inhibiting intratumoral PGE2 levels by 40%, had no antitumor activity alone or in combination with radiation. This suggests that COX-2-derived PGE2 may differ from that generated by COX-1, possibly due to microenvironment or distribution. The human head and neck model, 1483 HNSCC, allowed us to demonstrate that endogenous COX-2 and PGE2 levels can be elevated in response to radiotherapy. This observation suggested an important role in the stress response of the tumor after challenge with radiation. This is an important in vitro observation following the report of radiation-induced COX-2 expression in PC3 cells in vitro (6). We demonstrated the importance of the COX-2 product PGE2 by treating 1483 HNSCC tumors with a neutralizing antibody to PGE2 (2B5) and observing synergy with radiotherapy similarly to that observed with celecoxib. This suggests that COX-2-derived PGE2 is the primary survival agent that is removed by celecoxib. Using the HT29 model, we demonstrated that stromal COX-2 was a significant target of celecoxib after radiation insult, observing enhanced tumor control over either monotherapy alone. Because this tumor model only has COX-2 protein in the tumor stromal component, this suggests that tumor neovascularization may be a primary target of celecoxib after radiation insult. Because there was no apparent damage to the surrounding tissue, it can be concluded that, after radiation damage, COX-2-derived PGE2 may be a critical survival factor of aberrant or immature tumor neovascularity. This would support the in vitro observation that endothelial tube formation was reduced when COX-2 inhibitors are combined with radiation (48). The syngeneic Col26 was then used to explore the effects of radiation and celecoxib on tumor neovascularization. Using a high molecular weight contrast dye and MRI to visualize tumor vascular function and integrity, we observed that the combination of celecoxib and radiation led to a significant increase in vascular permeability by 24 h post treatment, whereas neither treatment alone impacted this parameter compared with vehicle-treated control animals. Hence, loss of COX-2-derived PGE2 in the context of radiation insult may lead to greater damage in the tumor neovascularization. Subsequent maintenance of inhibition of COX-2 activity and prostaglandin synthesis after the acute therapy may impair the neovascularization and re-growth of the tumor, consistent with the delay in regrowth observed with combined therapy compared with single agent alone. Interestingly, we also observed that celecoxib could be dosed 24 h after radiation treatment and still result in similar synergy as seen with prior administration (data not shown). Cohn et al. (51) saw maximal reductions in crypt cell survival whether indomethacin was dosed 1 or 24 h after initial radiation insult on normal mice. Houchen et al. (4) also observed that adding back the survival factor dimethyl PGE2 to mice 1 h after radiation suppressed the apoptotic created by radiation damage in crypt epithelial cells. Taken together, these data suggest that PGE2 is acting at a critical time in the recovery of cells after radiation damage. More research will be required to delineate the critical role of COX-2-derived PGE2 in this biology.

A working model that needs confirmation has been constructed to describe the synergy between celecoxib and radiotherapy (Fig. 6). In this model, COX-2 and its prostaglandin products (particularly PGE2) are critical for early angiogenesis during establishment of the tumor (up to 0.3 ml in volume; Refs. 15, 16, 45, and 46) after which other

Celecoxib Synergizes with Radiation: a Working Model

![Diagram of Celecoxib Synergizes with Radiation](image_url)
SYNERGY BETWEEN CELECOXIB AND RADIATION

factors may drive angiogenesis. However, after damage by radiotherapy, COX-2-derived prostaglandins become important survival factors for the immature tumor vasculature and possibly the tumor cells themselves. But in the presence of a COX-2 inhibitor such as celecoxib, debunking by IR will be greater due to the loss of protective COX-2-derived prostaglandins, particularly PGE₂ as suggested by the above data. Therefore, removal of these prostaglandins by COX-2 inhibition sensitizes these tissues to radiotherapy, leading to greater tumor debunking than is observed with radiation alone.

In conclusion, PGE₂ derived from COX-2 plays an important role in tumor survival after radiation damage. Celecoxib, as a selective COX-2 inhibitor, has the potential to safely and effectively enhance the benefit of radiotherapy by removing COX-2-derived PGE₂. There is a need to determine the mechanism(s) of tumor neovascular permeability and/or radiopotentiation, and clinical trials will be required to determine efficacy in humans.

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