Direct Evidence for a Role of Cyclooxygenase 2-derived Prostaglandin E\textsubscript{2} in Human Head and Neck Xenograft Tumors

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ABSTRACT

Both nonsteroidal anti-inflammatory drugs (NSAIDs) and cyclooxygenase (COX) 2-selective inhibitors such as celecoxib are being reported as having potent anticancer activity in laboratory models. Several reports have suggested that the mechanism of action of these agents in reducing tumor volume/burden is unrelated to their inhibition of prostaglandin synthesis. Many in vitro reports use supraphysiological concentrations of these drugs to demonstrate COX-independent activities on apoptosis or proliferation. In vivo, most murine tumor models express COX-2 only in the vasculature and stroma, unlike human tumors that also express COX-2 in the tumor cells. In general, these models have the limitation of having no measurable, COX-2-derived, prostaglandins, the inhibition of which correlates with antitumor efficacy. We report here that 1483 human head and neck xenograft tumors express COX-2 similar to the pattern observed in human solid tumors and that COX-2 activity produces high levels of prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). Inhibition of COX-2 by celecoxib resulted in loss of intratumor PGE\textsubscript{2} levels and reduced tumor growth in a dose-dependent manner. In contrast, a selective COX-1 inhibitor, SC-560, did not measurably reduce tumor prostaglandin levels or tumor growth despite the presence of COX-1 in the host and tumor cells. Celecoxib-treated tumors showed reduced proliferation and increased apoptosis of both tumor and stromal cells compared with vehicle controls. Specific inhibition of PGE\textsubscript{2} activity by a neutralizing antibody mimicked the reduced tumor growth observed after celecoxib treatment, suggesting growth is PGE\textsubscript{2} mediated. These data indicate that a major antitumor mechanism of action of celecoxib is inhibition of COX-2-derived prostaglandins, particularly PGE\textsubscript{2}, and suggest celecoxib as a novel therapeutic agent for human head and neck cancer.

INTRODUCTION

Inflammatory mediators such as cytokines, eicosanoids, and growth factors are thought to play a critical role in the maintenance, survival, and growth of tumor cells (1, 2). One major class of mediators, prostaglandins, is produced from arachidonic acid by an initial reaction with either of two enzymes, COX-1 or COX-2, followed by prostaglandin synthase. In particular, PGE\textsubscript{2}, COX-2, and microsomal PGE synthase have been linked with cancer (3). COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced in association with pathological inflammatory sites, including human cancers (1, 4). Both COX isozymes can be inhibited by traditional NSAIDs such as aspirin and ibuprofen. Studies have shown that regular intake of aspirin or other NSAIDs provides a 40–50% reduction in relative risk of death by colon cancer (5, 6). Other epidemiological studies demonstrate up to a 90% decreased risk of developing esophageal cancer by chronic use of aspirin (7, 8), suggesting that inhibition of COX in humans can have a chemopreventive effect (5). The importance of the COX-2 isoform in tumorigenesis was first demonstrated by the observation that, in rodent models of familial adenomatous polyposis, a genetic disease leading to GI cancer, loss of COX-2 activity by either genetic deletion or selective enzymatic inhibition suppressed intestinal polyp formation (9, 10). Celecoxib, a COX-2 inhibitor, has been approved in humans for adjunctive therapy in this population. Preclinical studies using selective COX-2 inhibitors have demonstrated chemopreventive activity in animal models of colon, bladder, and breast cancer (11–13). Additional evidence for the importance of COX-2 in tumorigenesis was reported by Hla et al. (14), who showed that selective COX-2 overexpression in the mammary gland of transgenic mice led to tumorigenesis. Taken together, these data provide strong evidence for the importance of COX-2 enzyme activity in oncogenesis and as a target of NSAID antitumor action.

COX-2 has been shown to be expressed in neoplastic epithelial cells in a wide variety of human tumor types (1, 15, 16). It has also been shown that different types of epithelial cancers produce high levels of PGE\textsubscript{2} (17, 18). Also, PGE\textsubscript{2} and its metabolite (13,14-dihydro-15-keto-PGE\textsubscript{2}) was reported to be elevated in the urine and plasma of cancer patients (19, 20). Analysis of human tumor tissue has demonstrated that PGE\textsubscript{2} levels can be elevated substantially over normal surrounding tissue (21). Additionally, human HNSCC has been reported to have elevated levels of COX-2 mRNA, protein, and PGE\textsubscript{2} compared with surrounding tissues (22–25). Zimmerman et al. (25) demonstrated that a COX-2-expressing HNSCC cell line produced 600 times more PGE\textsubscript{2} than a non-COX-2 expressing line and that inhibition of PGE\textsubscript{2} by two COX inhibitors resulted in reduced proliferation and increased apoptosis in vitro of the COX-2-expressing line. Similarly, Sumatani et al. demonstrated that COX-2 inhibition limited growth of an oral cancer cell line expressing high levels of COX-2. (26). Growth of another cell line, which expressed only low levels of COX-2, was not affected by treatment. Although, in this study, the levels of inhibitor reported were higher than would be expected to be necessary for COX-2 inhibition, similar results were obtained with COX-2 antisense oligonucleotides and growth was rescued by addition of exogenous PGE\textsubscript{2}.

In addition to neoplastic epithelium, COX-2 is highly expressed in the tumor microvasculature in many human tumors (1, 15, 16). This suggests that COX-2-derived PGE\textsubscript{2} may also support tumor growth through angiogenic endothelial cell growth and blood vessel formation (27, 28). This hypothesis is supported by the observation that celecoxib inhibits fibroblast growth factor-mediated angiogenesis in a rat corneal micropocket assay (29). We have previously reported that the COX-2 inhibitor, celecoxib, inhibits tumor growth in a number of murine tumor models. However, in these models, COX-2 could only be detected in the neovascularure and not the tumor cells. Because COX-2-derived PGE\textsubscript{2} synthesis is not measurable in these models, it has been speculated that COX-2 inhibitors may act independently of inhibition of prostaglandin synthesis. This controversy has been fostered by reports of COX-independent activities of traditional NSAIDs, particularly in vitro at high micromolar concentrations (30–32). We hypothesize that the primary mechanism of action of celecoxib within these models is believed to be antiangiogenic (16, 29, 33) elegantly supported by Williams et al. (34), who showed that host COX-2 is...
important for tumor neovascularization and subsequent tumor growth. However, because most human cancers express COX-2 in both the neoplastic and the stromal cells, the current models do not fully reflect the complexity of human cancer (16). In this paper, we demonstrate that COX-2-derived PGE2 drives tumor growth in a xenograft model of HNSCC with COX-2 expression that occurs in both stromal (inflammatory and neovascular) and neoplastic cells and has measurable COX-2-derived prostaglandin levels. Inhibition of PGE2 synthesis with celecoxib or neutralizing PGE2 activity with an antibody to PGE2 inhibited tumor growth in this model, suggesting that tumor growth is PGE2 dependent. Furthermore, inhibition of PGE2 production by celecoxib is correlated with increased apoptosis and decreased proliferation in both tumor and stromal cells.

MATERIALS AND METHODS

1483 Cell Culture. Human HNSCC 1483 cells were derived from the retromolar trigone from an untreated patient with nodal metastasis (35). Cells were grown in standard tissue culture conditions in DMEM/F-12 media (Life Technologies, Inc.) supplemented with 1% HEPES buffer, 2 mm l-glutamine, pyridoxine hydrochloride, 10% FBS, and 0.05 mg/ml gentamicin (Life Technologies, Inc.). Cells were fed every other day, passaged at 80–90% confluence and not maintained beyond passage 10.

In Vivo Tumor Studies. HNSCC 1483 cells (1 × 10^6) suspended in 30% Matrigel (BD Biosciences) in HBSS (Life Technologies, Inc.) were implanted in both tumor and stromal cells.

Celecoxib Inhibits 1483 Tumor Growth and PGE2 Production In Vivo. To study the role of COX-2 and prostaglandins in tumor growth, several cancer cell lines were implanted into mice and, after established tumor growth, were analyzed for known features of human cancer. Of nine cell lines studied in vivo, including LNCAp, Lewis lung, and B16F10, all of the tumors cells expressed COX-1 but not COX-2, with the exception of COX-2 expression in the angiogenic vasculature. In contrast, primary cultures of 1483 cells, derived from a human HNSCC, expressed COX-2 in vitro, were tumorigenic, and maintained COX-2 expression and activity in vivo (Fig. 1). 1483 tumors maintained very high levels of PGE2 compared with the normal left paw. Interestingly, the expression pattern of COX-2 was similar to that observed in human head and neck cancer (Refs. 22, 25; Fig. 1, A and B). In tumors, clusters of neoplastic epithelial cells having moderate to strong COX-2 expression and stromal cells with having moderate to strong COX-2 expression and stromal cells with COX-2-DERIVED PGE2 PROMOTES TUMOR GROWTH
cally, animals in the vehicle-treated control groups developed tumors ranging from 0.62 to 1.46 ml in ~27 days (Fig. 2). Treatment starting at 0.1 ml with celecoxib, a COX-2 inhibitor, reduced tumor growth in a dose-dependent manner. At day 27, celecoxib at 40 and 160 ppm inhibited tumor growth by 57 and 78% of vehicle controls, respectively (Fig. 2A). This inhibition of tumor growth was associated with a 62 and 91% decrease in the levels of tumor PGE2 (Fig. 2A). In contrast, celecoxib at the low dose of 10 ppm did not inhibit tumor growth and only decreased tumor PGE2 levels by a modest 35% (Fig. 2A). The role of COX-1 in tumor growth was examined with a selective COX-1 inhibitor, SC-560. At 20 ppm, a dose known to inhibit COX-1, but not COX-2 enzyme SC-560, had no effect on tumor growth or tumor PGE2 levels (Fig. 2B). In contrast, tumor growth was inhibited by 81 and 91% when celecoxib was administered at doses of 160 and 1600 ppm, respectively (Fig. 2B). Furthermore, doses of 160 and 1600 ppm of celecoxib inhibited tumor PGE2 by 67 and 83%, respectively (Fig. 2B). Thus, inhibition of COX-2 by celecoxib and not COX-1 resulted in tumor inhibition. Also, celecoxib treatment did not alter COX-1 or COX-2 protein levels measured by Western blot analyses (data not shown).

In Vivo Selectivity of COX Inhibitors. In vivo selectivity of each COX inhibitor was evaluated in serum from treated animals by immunoassaying TXB2 levels, the degradation product of TxA2 formed in the platelet by COX-1. Inhibition of tumor growth and PGE2 by celecoxib was not associated with platelet COX-1 at any dose. SC-560, however, inhibited COX-1-derived TXB2 by 84% compared with vehicle controls and normal nontumor bearing mice (normal, Fig. 3). These data indicate that celecoxib selectively inhibited COX-2 activity and PGE2 production in 1483 tumors and that inhibition of PGE2 correlated well with the inhibition of tumor growth.

1483 Tumor Growth Is PGE2 Dependent. The role of PGE2 in the growth of 1483 tumors was additionally examined in tumor-bearing mice treated with a PGE2-neutralizing mouse monoclonal antibody (2B5; Ref. 38). After a dosing regimen previously described (39), 2B5 antibody treatment resulted in a similar inhibition of tumor growth as observed with celecoxib (93 versus 84% of vehicle treated control; Fig. 4). The MOPC31 IgG isotype control did not reduce tumor growth. Although the 2B5 antibody was an effective treatment for tumor control, dosing had to be discontinued at day 21 because of gastrointestinal toxicity, leading to body weight loss and death. Thus, the treatment with the COX-2 inhibitor celecoxib was able to inhibit COX-2-derived PGE2 while sparing constitutive COX-1-derived PGE2 necessary for normal gastrointestinal homeostasis.

Fig. 1. COX-2 expression in (A) a human head and neck cancer and (B) 1483 human head and neck xenograft tumor. COX-2 expression was observed in clusters of neoplastic epithelial cells throughout the tumor. COX-2 expression was also observed in some but not all stromal cells. Colocalization of (C) COX-2 and endothelial cell marker (D) CD31 in 1483 tumor. Image overlay of (E) CD31 and COX-2 illustrate coexpression (orange) in some endothelial cells. Other stromal cells negative for CD31 also expressed COX-2 (data not shown). On the basis of fluorescence intensity, COX-2 expression in endothelial cells was ~10-fold lower than in tumor cells. Bar = 25 μm.

Fig. 2. Effect of cyclooxygenase inhibitors on 1483 xenograft tumor growth and PGE2 production. Vehicle-treated 1483 tumors exhibited a robust exponential growth starting at day 7. Celecoxib treatment at 10–160 ppm (A) and 1600 ppm (B) produced a dose-dependent inhibition in growth and PGE2 content after 28 days. B, a selective COX-1 inhibitor, SC-560, at a dose that significantly inhibits serum platelet COX-1 but not COX-2 (20 ppm) had no effect on tumor growth. Celecoxib dosed dependently inhibited this production, whereas SC-560 had no effect. Data represent the mean ± SE (n = 8 mice/group); *, P < 0.05 compared with vehicle treated.
COX-2-derived PGE₂ promotes tumor growth

**Fig. 3. Effect of cyclooxygenase inhibitors on platelet COX-1-derived serum thromboxane production as measured by standard EIA.** Serum thromboxane production by platelet COX-1 was markedly inhibited by SC-560 but unaffected by celecoxib. These data illustrate the selectivity of celecoxib for COX-2 in these studies and illustrate the important role of COX-2 compared with COX-1 in 1483 tumor growth. Data represent the mean ± SE (n = 8 mice/group); *, P < 0.05 compared with vehicle treated.

**Fig. 4. Effect of anti-PGE₂-neutralizing antibody on 1483 tumor growth.** i.p. injections of 2B5 mouse anti-PGE₂ antibody, 10 mg/kg, 3 times/week, was as efficacious as celecoxib (160 ppm) in inhibiting 1483 tumor growth. Continued dosing, however, was toxic and produced GI lesions and death in all animals after 14 days of dosing. Control IgG, MOPC31, at equivalent doses had no effect on tumor growth or animal health. Data to inhibit proliferation and associated stromal cell proliferation by 51 and 56%, respectively, corresponding to a decrease in the proliferation index from 11.3 to 5.5% and 6.3 to 2.8%, respectively (Fig. 5). Similar effects on proliferation were observed on tumors treated with 160 ppm celecoxib (data not shown). Apoptosis, measured by TUNEL staining, was increased by celecoxib in the tumors cells by 57%, increasing the apoptosis index from 2.9 to 6.7%. The stromal cell apoptotic index increased from 2.4 to 4.7%, an increase of 49% (Fig. 5). Inhibition of COX-1 by SC-560, however, did not significantly impact either of these parameters (data not shown).

**Effect of COX-2-derived PGE₂ on Normal Proliferating Epithelium.** To address the role of COX-2 in normal proliferating cells, a COX-2 inhibitor (celecoxib), a nonselective NSAID (indomethacin), and a cytotoxic agent (5-FU) were tested in their ability to inhibit proliferation of ileum epithelium in the crypts. Vehicle, celecoxib (160 and 1600 ppm), indomethacin (2 mg/kg/day), and 5-FU (40 mg/kg/day) were administered for 4 days in nude mice, and 24 h after the last administration of 5-FU, mice were injected with BrdUrd and sacrificed 2 h later. Proliferation of normal cells (as represented by ileum crypt BrdUrd incorporation) was not affected by either dose of celecoxib, whereas proliferation of intestinal epithelium in animals treated with indomethacin or 5-FU was inhibited by 36 and 77%, respectively (Fig. 6).

**DISCUSSION**

Prostaglandins have long been considered important in tumor growth and development. However, reducing prostaglandin levels within tumors by use of nonselective NSAIDs in conjunction with a chemotherapeutic or radiation therapeutic regimen would likely be limited because of toxicity that could occur to proliferating tissues such as the GI tract. Discovery of distinct isoforms of cyclooxygenase led to the notion that selective inhibition of COX-2 would avoid the gastric toxicity associated with inhibition of both isoforms. The observation that COX-2 is highly expressed in a majority of human tumors suggests that COX-2 inhibition may be a useful therapeutic mechanism for inhibiting tumor progression and growth of human cancer (22, 40–44). A role for COX-2 in cancer is additionally supported by the observation that genetic deletion of host COX-2 results in reduced tumor generation and growth rates (34) and that tissue-specific overexpression of COX-2 leads to tumor formation in transgenic mice (14). These data additionally support the hypothesis

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In Vivo Inhibition of COX-2-derived PGE₂ Results in Apoptosis and Reduced Proliferation in 1483 Tumors. The effects of COX-2 inhibition on tumor epithelial and endothelial cell proliferation and apoptosis were examined in vivo. Celecoxib (160 and 1600 ppm) or SC-560 (20 ppm) was administered in the food to 1483 tumor-bearing (0.4 ml) mice for a total of 12 days. On day 12, compound and vehicle treated mice were injected i.p. with BrdUrd and sacrificed 2 h later. BrdUrd incorporation, an index marker of cellular proliferation, was determined in both the tumor cells and associated stromal cells (Fig. 5). Celecoxib treatment inhibited tumor and stromal cell proliferation by 51 and 56%, respectively, corresponding to a decrease in the proliferation index from 11.3 to 5.5% and 6.3 to 2.8%, respectively (Fig. 5). Similar effects on proliferation were observed on tumors treated with 160 ppm celecoxib (data not shown). Apoptosis, measured by TUNEL staining, was increased by celecoxib in the tumors cells by 57%, increasing the apoptosis index from 2.9 to 6.7%. The stromal cell apoptotic index increased from 2.4 to 4.7%, an increase of 49% (Fig. 5). Inhibition of COX-1 by SC-560, however, did not significantly impact either of these parameters (data not shown).

**Fig. 5. Effect of celecoxib treatment on tumor and stromal cell proliferation and apoptosis in vivo.** Using BrdUrd immunohistochemistry (A and B) to assess proliferation, celecoxib consistently inhibited proliferation in tumor cells (upper right graph) and stromal cells (upper left graph) by 50–60% after 12 days of treatment (*, P < 0.0002 and P < 0.02, respectively). Using TUNEL to assay apoptosis in tumor sections (C and D), celecoxib also induced a consistent 2–3-fold increase in tumor cell (lower right graph, *, P < 0.01) and stromal cell (lower left graph) apoptosis. SC-560, the selective COX-1 inhibitor had no effect on either parameter (data not shown).
neutralizing anti-PGE2 antibody that mimicked celecoxib therapy. The advantage of celecoxib, however, is its lack of GI toxicity. These findings further suggest that celecoxib may provide a means for safe and effective anticancer therapy and clearly link this antitumor efficacy to inhibition of COX-2 enzymatic activity and PGE2 inhibition. Clinical trials with celecoxib in human cancer will be required to prove clinical benefit.

REFERENCES


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