Antiangiogenic and Antitumor Activities of Cyclooxygenase-2 Inhibitors


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ABSTRACT

We provide evidence that cyclooxygenase (COX)-2-derived prostaglandins contribute to tumor growth by inducing newly formed blood vessels (neangiogenesis) that sustain tumor cell viability and growth. COX-2 is expressed within human tumor neovasculature as well as in neoplastic cells present in human colon, breast, prostate, and lung cancer biopsy tissue. COX-1 is broadly distributed in normal, as well as in neoplastic, tissues. The contribution of COX-2 to human tumor growth was indicated by the ability of celecoxib, an agent that inhibits the COX-2 enzyme, to suppress growth of lung and colon tumors implanted into recipient mice. Mechanistically, celecoxib demonstrated a potent antiangiogenic activity. In a rat model of angiogenesis, we observe that corneal blood vessel formation is suppressed by celecoxib, but not by a COX-1 inhibitor. These and other data indicate that COX-2 and COX-2-derived prostaglandins may play a major role in development of cancer through numerous biochemical mechanisms, including stimulation of tumor cell growth and neovascularization. The ability of celecoxib to block angiogenesis and suppress tumor growth suggests a novel application of this anti-inflammatory drug in the treatment of human cancer.

INTRODUCTION

Inflammatory mediators such as cytokines, eicosanoids, and growth factors are thought to play a critical role in the initiation and maintenance of cancer cell survival and growth (1). One of these mediators, PGE2, is produced in large amounts by tumors. PGE2 is produced from arachidonic acid by either of two enzymes: COX-1 or COX-2. COX-1 is constitutively expressed in most tissues, whereas COX-2 is induced in inflammatory cells and in human tumors by cytokines and tumor promoters (2–5). Both COX isozymes can be inhibited by traditional NSAIDs, such as aspirin and indomethacin. Several studies show that regularly taking aspirin or other conventional NSAIDs provides a 40–50% reduction in relative risk of death by colon cancer, indicating that inhibition of COX in humans has a chemopreventive effect (6). In rodent models of FAP, a genetic disease leading to colon carcinoma, blockade of COX-2, either by gene deletion or by pharmacological inhibition of enzyme activity, suppresses intestinal polyp formation (7). COX-2 inhibition also demonstrates chemopreventive activity against colon carcinogenesis (8). Taken together, these data provide strong evidence for the importance of COX-2 enzyme activity in oncogenesis.

One of the mechanisms by which PGE2 supports tumor growth is by inducing the angiogenesis necessary to supply oxygen and nutrients to tumors >2 mm in diameter (9, 10). We have observed COX-2 expression in newly formed blood vessels within tumors grown in animals, whereas under normal physiological conditions the quiescent vasculature expresses only the constitutive COX-1 enzyme (11). This novel observation together with studies showing the expression of the enzyme in cancer cells suggests a predominant role for COX-2 in tumor regulation and angiogenesis. In this study, we evaluated the expression of COX-2 in different types of human cancers and, in particular, the expression of COX-2 in tumor angiogenesis. Using inhibitors of COX-2 and COX-1, we demonstrated that COX-2-derived PGs mediate tumor growth and metastasis in two independent animal models and in FGF-2-induced angiogenesis. Celecoxib demonstrated potent antiangiogenic and antitumor activity in vivo, suggesting the potential use of this anti-inflammatory drug in the treatment of human cancer.

MATERIALS AND METHODS

Immunohistochemistry Analysis. Cancer tissues were obtained from archival samples from Weill Medical College of Cornell University (New York, NY) and from Washington University (St. Louis, MO). The study was approved by the Committees on Human Rights in Research at these institutions. Paraffin-embedded sections were cut into 4-micron sections, mounted onto polylysine-coated slides, dewaxed in xylene, rehydrated in alcohol, and blocked for endogenous peroxidase (3% H2O2 in methanol) and avidin/biotin (Vector Blocking Kit). Sections were treated with TMB-BB [0.1 M Tris (pH 7.5), 0.15 M NaCl, 0.5% blocking agent, 0.3% Triton-X, 0.2% saponin], and incubated with a 1:500 dilution of COX-2-specific antibody (PG-27; Oxford Biomedical Research Inc.). Specificity of the antibody was determined by using control sections that were incubated with antisera in the presence of a 100-fold excess of human recombinant COX-2 protein, or with isotype-matched IgG normal rabbit serum. Immunoreactive complexes were detected using tyramide signal amplification (TSA-indirect) and visualized with the peroxidase substrate, AEC. Slides were counter stained with hematoxylin.

All slides were evaluated by a minimum of two board certified pathologists, one of whom specializes in the specific cancer type.

Lewis Lung Carcinoma. Lewis Lung Carcinoma cells (1 × 106) were implanted into the paws of C57/B6 male mice and divided into five treatment groups of 20 animals each. Group 1 received normal chow, whereas the other groups received celecoxib in the diet, from date of implant until end of study, at doses between 160-3200 ppm. All of the animals in the control group developed tumors reaching the size of 1.5–2.0 mm in approximately 35 days. Tumor volume was determined biweekly using a plethysmometer. At the end of the study, the animals were sacrificed and the tumor and lungs were excised. Analysis of lung metastasis was done in all of the animals by counting surface metastatic lesions under stereomicroscope and verified by histochemical analysis of consecutive lung sections.

HT-29 Human Colon Tumor in Nude Mice. HT-29 human colon carcinoma cells were also implanted in the hind paws (1 × 106 cells) of nude mice (n = 15/treatment group). Celecoxib therapy was initiated in the diet at doses between 160-1600 ppm when tumors reached a mean volume of 100 mm3 and maintained for the duration of the experiment. At the end of the experiment, the lungs were excised, fixed in Streeks (Streeks Laboratory, Omaha, NE), and the total number of neoplastic nodules present in the lung surface were counted as described previously.

Corneal Angiogenesis Model. An intrastromal pocket was surgically created in the cornea of an anesthetized rat. A slow release hydron/sucralfate pellet containing either 100 ng of PGE2 or saline (placebo) was inserted into the pocket approximately 1.4 mm from the temporal corneal limbus. The pocket was self-sealing, and antibiotic ointment was placed on the eye to prevent infection. COX inhibitors were administered by gavage in a 0.5-ml suspension of 0.5% methylcellulose (Sigma Chemical Co., St. Louis, MO), 0.025% Tween 20 (Sigma Chemical Co.) twice daily at 12-h intervals, beginning the day before surgery and continuing the length of the study. Four days after surgery, the corneas were examined under a slit lamp microscope and the

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The abbreviations used are: PGE2, prostaglandin E2; NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PGE2, basic fibroblast growth factor; FAP, familial adenomatous polyposis.
neovascular response was quantified by measuring the average new vessel length (VL), the corneal radius \( r = 2.6 \) mm, and the contiguous circumferential zone \( CH = \) clock hours where \( 1 \) CH \( = 30 \) degrees, and applied to the formula: area \( (\text{mm}^2) = (\text{CH}/12 \times 3.14(r^2-(r-VL})^2) \) (2). All animal treatment protocols were reviewed by and were in compliance with Monsanto’s Institutional Animal Care and Use Committee.

Statistics. The data are expressed as the mean +/- SEM. Student’s and Mann-Whitney tests were used to assess differences between means using the InStat software package. Significance was accepted at \( P < 0.05 \).

RESULTS

Immunohistological Localization Of COX-2 in Human Tumor Angiogenesis. We used a highly sensitive immunohistochemical method to carefully examine incidence and distribution of COX-2 in the major human cancers, including its expression in tumor angiogenesis. Immunohistological analysis revealed COX-2 is consistently expressed in the neoplastic lesions in colon (17 COX-2-positive neoplastic colons/20 neoplastic colons examined), prostate (20 of 20), lung (19 of 20), and breast (16 of 18) cancers (Fig. 1). Moderate-to-strong COX-2 expression was detected in approximately 40–80% of the total neoplastic cells in most tumors, and moderately and well-differentiated carcinomas showed significantly higher COX-2 immunoreactivity than poorly differentiated cases. In addition to expression of COX-2 within neoplastic cells per se, COX-2 was also detected in the angiogenic vasculature present within the tumors and preexisting vasculature adjacent to cancer lesions (Fig. 1). In contrast, COX-2 was not detected in normal colonic epithelium or stroma, with the exception of a small number of colonic crypt epithelial cells in which the enzyme was weakly detected (Fig. 2A). Interestingly, during colon oncogenesis COX-2 is expressed not only in the neoplastic adenomas in patients with FAP (Fig. 2B), in colon cancer (Fig. 1), and robustly in metastatic lesions in liver (Fig. 2C), but also in the neovasculature associated with the adenomas (Fig. 2B), colonic lesions (Fig. 1), and metastatic liver lesions (Fig. 2D). The expression of COX-2 in the neovasculature of human tumors seems to be a general characteristic of epithelial tumors. In addition to the cancers mentioned above, COX-2 was observed in the angiogenic vessels in most of the human cancers analyzed thus far, including head and neck and pancreas (12,
COX-2 INHIBITION: ANTITUMOR AND ANTIANGIOGENIC EFFECTS

Fig. 2. COX-2 expression in colon oncogenesis. A. COX-2 is generally not found in normal tissues, with the exception of weak expression in some colonic crypt epithelial cells (red, small arrows). B. COX-2 is clearly expressed in the adenomas of FAP patients (red, FAP arrows) and in the blood vessels associated with the adenomas (red, BV arrows). C. Striking homogeneity and robust levels of COX-2 immunoreactivity were detected in metastatic lesions in liver in eight of eight colon cancer patients with metastatic disease (red). D. COX-2 was also clearly detected in the vasculature within metastatic tissues and adjacent to neoplastic clusters (red). Similar results were observed in patients with pancreatic cancer, cholangiocarcinoma, and Kaposi’s sarcoma.

13). These results imply COX-2 may play a functional role in tumor-induced angiogenesis and subsequent progression to metastatic disease and may partially explain the potent antimetastatic activity of COX-2 inhibitors observed in rodent models.

In contrast to the COX-2 expression in human cancer tissues, COX-1 is expressed in both normal and neoplastic regions in all tissues, and seemed to be particularly expressed in the tumor stroma that included fibroblasts, smooth muscle cells, and the vasculature. The broad distribution of this isoform in both normal and neoplastic tissues makes it difficult to determine whether changes in COX-1 expression occurred during tumorigenesis (14, 15).

Inhibition of Tumor Growth and Lung Metastasis by Celecoxib in Mice. The COX-2 inhibitor celecoxib, a 1,5 diarylpyrazole with >300-fold selectivity for COX-2 versus COX-1 (16, 17) in vitro, was tested in two animal models of lung and colon cancer to evaluate its effect on tumor growth and lung metastases. Lewis Lung carcinoma cells implanted into the right paws of a series of 20 C57/B16 male mice develop tumors reaching the size of 1.5–2.0 cm³ in approximately 35 days. Celecoxib supplied in the diet continuously from date of implant at doses between 160-3200 ppm significantly retarded the growth of these primary tumors (Fig. 3A). The inhibitory effect of celecoxib was dose dependent and ranged from 48–85% when compared with untreated tumors (p < 0.001, Student’s t test). In the same model, cyclophosphamide (50 mg/kg, i.p., days 5, 7, and 9) produced a 34% reduction in tumor volume. A pharmacological effect of celecoxib on lung metastasis was evaluated in all of the animals by counting the number of metastases in a stereomicroscope and by histochemical analysis of consecutive lung sections. Celecoxib did not affect the number and size of lung metastases at the lower dose of 160 ppm, however, the number of metastatic nodules was reduced by >50% in animals treated with doses between 480 and 3200 ppm. Moreover, histopathological analysis revealed that celecoxib dose-dependently reduced the size of the metastatic lesions in the lung (data not shown).

Celecoxib was also tested in nude mice that had been implanted with the human colon cancer cell line HT-29. These tumors reached 1.5–2.0 cm³ in size by 40–55 days after implantation. Celecoxib was introduced to the diet at doses ranging from 160-1600 ppm when the tumors had reached 0.1 cm³ in volume. Maximal inhibition of 67% of tumor growth was achieved with the lowest dose of celecoxib (Fig. 3B). In addition, celecoxib dose-dependently inhibited tumor lung metastases (Table 1, Fig. 3C). The inhibitory effect was remarkable with a 65% inhibition at 160 ppm and a maximal effect of 91% at 1600 ppm. Celecoxib peak plasma levels were approximately between 0.2 and 9.0 µg/ml for the 160 and 3200 ppm, respectively. The peak plasma concentrations achieved in the studies were similar to those reported by Reddy et al. (8) in rats, in which a potent chemopreventive properties of celecoxib in colon carcinogenesis was observed. Similar to the study by Reddy et al. (8), no toxicity was observed in any of the animals as measured by weight gain/loss, as well as gross pathological examination of the gastrointestinal tract of the animals at necropsy.

Effect of COX Inhibitors on FGF-induced Corneal Angiogenesis. The contribution of COX-2 to angiogenesis was evaluated using an in vivo rat corneal model (18). The pharmacological effects of the COX-2 inhibitor celecoxib, which at therapeutic doses in humans does not inhibit COX-1, the COX-1 inhibitor SC-560 and the conventional NSAID (COX-1/COX-2) indomethacin (16, 17) were measured. Celecoxib caused a substantial reduction in the number and length of sprouting capillaries (Fig. 4A). Celecoxib dose-dependently inhibited the angiogenic response with an ED₅₀ of 0.3 mg/kg/day, and with maximal inhibitory activity of 80% at a dose of 30 mg/kg/day (Fig. 4B). Plasma levels were determined 4 h after the last dose and found to be approximately 0.3 and 2.0 µg/ml for the 3 and 30 mg/kg/day, respectively. Indomethacin, an inhibitor of both COX-1 and COX-2, also inhibited angiogenesis with an inhibition of 60% at the near maximally tolerated dose of 1 mg/kg/day (Fig. 4B). Inhibition of angiogenesis in rats dosed with indomethacin at 3 mg/kg/day was not determined due to the peritonitis, and subsequent death of these rats before the 4th day of the study. In contrast to the effects of celecoxib and indomethacin, the COX-1-specific inhibitor SC-560 did not affect angiogenesis when given at doses of 3 and 10 mg/kg/day. To determine the specificity of the inhibitors in vivo, sera from the same rats were immunoassayed for TxB₂, a product of platelet COX-1 activity. While potent inhibiting angiogenesis, celecoxib did not diminish platelet COX-1 activity in blood taken from the same animals (Fig. 4, B and C). Because indomethacin inhibits both isozymes at doses that suppressed angiogenesis, it inhibited platelet COX-1 activity, as well (Fig. 4, B and C).
COX-1 inhibitor SC-560 inhibited platelet COX-1-derived TXB$_2$ by 89% and 97% at doses of 3 and 10 mg/kg/day, respectively, without any effect on the angiogenic response in the cornea (Fig. 4, B and C). To determine whether the antiangiogenic activity of celecoxib was due to the inhibition of PG synthesis, we tested an inactive isomer (1,3- versus 1,5-diarylpyrazole) of celecoxib. This compound was completely devoid of antiangiogenic activity at a maximal dose of 30 mg/kg/day (data not shown).

Histological examination of the angiogenic cornea revealed the presence of new angiogenic blood vessels and several types of COX-2-positive cells (Fig. 4, E and F). No angiogenesis was observed in corneas implanted with placebo (Fig. 4D). In contrast, corneas implanted with the growth factor FGF-2 induced a strong neovascularization that is accompanied by corneal thickening with an expanded stroma filled with an influx of COX-2-positive cells, including fibroblasts, macrophages, and endothelial cells (Fig. 4, E and F). Whereas endothelial cells present in the established limbic vessels expressed the COX-1 enzyme, newly vascularized endothelium expressed COX-2 (Fig. 4, E and F).

In agreement with the studies of Kenyon et al. (18), the angiogenic response we studied seemed to be mediated directly by FGF-2 and was not due to an inflammatory reaction to the implants. Indeed, the insertion of placebo pellets induced an initial inflammatory response similar to the FGF-2-containing pellets; however, this inflammation resolved within the first 24 h without a subsequent formation of new blood vessels (Fig. 4D).

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<th>Celecoxib (ppm)</th>
<th>Lung metastasis (average ± SE)</th>
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<tr>
<td>0</td>
<td>55 ± 17</td>
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<tr>
<td>160</td>
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DISCUSSION

There is ample evidence to suggest an important role for COX-2 in cancer. This newly discovered enzyme is not normally present under physiological conditions in most tissues, however, it is rapidly induced by a variety of cytokines and mitogens (2–5). Many reports indicate that COX-2 is up-regulated in most human tumors (12–15, 19–21). We extended this observation to examine the expression of COX-2 in more than 150 samples of different types of human cancers. We consistently find COX-2 expression in most cancer tissues, including colon, lung, breast, and prostate. Furthermore, we can clearly detect the presence of COX-2 in the angiogenic vasculature in most of the tumors analyzed. Another important finding of this study was the detection of COX-2 in the angiogenic blood vessels present in livers from patients with metastatic colon carcinoma (Fig. 2). These novel findings suggest COX-2 may play a role in tumor angiogenesis and suggest that inhibitors of this pathway may affect tumor growth by inhibiting the formation of blood vessels necessary for tumor survival (10).

Two animal models, the Lewis lung carcinoma and the human colon carcinoma HT-29, were selected to evaluate the antitumorigenic effects of celecoxib. Both models develop primary tumors as well as lung metastases when the tumors reach volumes larger than 1.5 cm$^3$ in size. Celecoxib dose-dependently inhibited tumor growth and the number and size of lung metastases. These data confirm the efficacy of the COX-2 inhibitor in blocking the growth of the primary tumor, as well as its antimetastatic potential. The anticancer efficacy observed in both animal models with celecoxib was devoid of any signs of gastrointestinal toxicity that is typical of nonselective NSAIDs and preclude their use in cancer therapy. Interestingly, the expression of COX-2 in the animal models of cancer was mainly restricted to the angiogenic blood vessels, the preexisting vasculature adjacent to the primary tumor, the blood vessels invading the metastatic lesions in the lungs of these animals, and not in the tumor cells themselves. On the basis of these findings, we suggest that COX-2-derived PGs contribute to tumor growth in these rodent models by inducing newly formed blood vessels that sustain tumor cell viability and growth. Furthermore, the results suggest that a potent antiangiogenic activity of celecoxib seems to be the primary mechanism of action in these animal models of cancer and results in substantial inhibition of tumor growth and metastasis.
To test this hypothesis, we evaluated the antiangiogenic activity of celecoxib in the rat corneal model of angiogenesis. We found that celecoxib, a compound with 300-fold in vitro selectivity for COX-2 versus COX-1 (5) was a very potent inhibitor of angiogenesis with an ED$_{50}$ around 0.3 mg/kg/day, a dose in the range of efficacy against inflammation (8, 16). The effect of celecoxib was dose-dependent and specific for the target enzyme because, at all doses tested, it did not inhibit serum TXB$_2$ derived from platelet COX-1. The potent antangiogenic effect of celecoxib seems to be derived from its capacity to inhibit PG production via COX-2 because neither an inactive isomer of celecoxib, nor a COX-1 inhibitor was able to block the development of angiogenesis in the rat cornea. Indomethacin, a potent inhibitor of both COX-1 and -2, inhibited both angiogenesis and platelet COX-1 activity, and at higher doses caused gastrointestinal toxicity and death. Taken together, these results suggest that PGs produced by FGF-2 induction of COX-2 are essential to neovascularization. These new findings contrast somewhat with a recently published study by Tsujii et al. (22). Using a coculture system of endothelial cells with colon cancer cells they report that COX-1, but not COX-2, is the enzyme necessary for the endothelial cells to form tubes in vitro. Tube formation by capillary endothelial cells in vitro may closely reflect quiescent established capillaries in vivo that, in fact, express COX-1.

Fig. 4. Celecoxib is a potent inhibitor of angiogenesis. A, a representative photograph of neovascularization induced by basic FGF-2 in the rat cornea (left) and its inhibition by celecoxib (right). B, pharmacological inhibition of angiogenesis by COX inhibitors. The COX-2 inhibitor celecoxib, the COX-1-specific inhibitor SC-560, and the nonspecific COX inhibitor indomethacin were dosed p.o. b.i.d. ($n=6$ per dose) for 5 days. Celecoxib and indomethacin inhibited angiogenesis ($P<0.001$, Student's $t$ test) at all doses tested, whereas SC-560 had no effect. C, representation of COX-1 activity in vivo. Sera from the same rats were immunoassayed (Cayman Chemical) for TXB$_2$, a product of platelet COX-1 activity. While potently inhibiting angiogenesis, celecoxib had no effect on platelet COX-1 activity. SC-560 did not affect angiogenesis at 3 and 10 mg/kg/day, doses that clearly inhibited COX-1 by 68% and 86%, respectively. Because indomethacin inhibits both isozymes, at doses that suppressed angiogenesis, it inhibited platelet COX-1, as well. These experiments were repeated at least three times with six rats per group. D, COX-2 expression (red) and immunohistological examination of a transverse section of the temporal limbic region of the rat cornea 4 days after implant with a placebo pellet. The eyes were resected, fixed in 10% formalin, and stained for COX-2 using specific antisera. The photograph shows the presence of COX-2-positive macrophages (M) near the limbic vessels (L). No evidence of inflammation or neovascularization was observed in placebo corneas. E, an identical region of the FGF-2 implanted cornea showed an expanded stroma filled with an influx of COX-2-positive cells, including macrophages (M), fibroblasts, and endothelial cells. Mature endothelial cells present in the established limbic vessels (L) did not express COX-2. F, high-power photomicrograph of a section of corneal stroma showed the presence of newly developed angiogenic blood vessels (arrows) with some luminal RBCs. The endothelium of these vessels expressed the inducible COX-2 enzyme.
vasculature. Thus, in human cancer, inhibition of COX-2 may prove effective in two ways: by blunting the tumor cell production of angiogenic growth factors and by inhibiting the growth of the neovascular cells themselves.

The contribution of inflammatory mediators to the angiogenesis of tumors and their growth is becoming evident. Our observation that FGF-2-induced angiogenesis is dependent on the expression of COX-2 strongly suggests that COX-2 plays an important role in the generation of tumor blood supply. This was further demonstrated with the presence of COX-2 in the neovascularization of most human tumors and in metastases such as in the liver. On the basis of the role of COX-2 in angiogenesis, together with the expression of the enzyme in human tumors and the remarkable efficacy observed by inhibitors of this pathway in animal models, we postulate that inhibition of COX-2 should produce antiangiogenic and antitumor results in the clinic. This hypothesis is further supported by studies indicating a role for COX-2 in the production of angiogenic factors by colon cancer cells (22), increased cell proliferation (23), prevention of apoptosis (24), increased metastatic potential (25), and the inhibition of immune surveillance (26). Finally, anti-inflammatory treatment using indomethacin, a nonselective inhibitor of COX-1 and COX-2, or prednisolone, a steroid known to inhibit the synthesis of COX-2 protein, prolonged survival in patients with metastatic solid tumors (27). In light of these data, a clinical trial designed to assess the use of celecoxib in the therapy of human cancer seems justified.

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