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Advances in Brief

Cyclooxygenase-2 Inhibition by Celecoxib Reduces Proliferation and Induces Apoptosis in Angiogenic Endothelial Cells *in Vivo*

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Abstract

Cyclooxygenase-2 (COX-2) is expressed within neovascular structures that support many human cancers. Inhibition of COX-2 by celecoxib delays tumor growth and metastasis in xenograft tumor models as well as suppresses basic fibroblast growth factor 2 (FGF-2)-induced neovascularization of the rodent cornea. The present studies were undertaken to evaluate possible mechanisms of the antiangiogenic and anticancer effects of celecoxib. Prostaglandin E_2 (PGE_2) and thromboxane B_2 (TXB_2) were increased in rat corneas implanted with slow-release pellets containing FGF-2 (338.6 ng of PGE_2/g and 17.53 ng of TXB_2/g) compared with normal rat corneas (63.1 ng of PGE_2/g and 2.0 ng of TXB_2/g). Celecoxib at 30 mg/kg/day p.o. inhibited angiogenesis (78.6%) and prostaglandin production by 78% for PGE_2 (72.65 ng/g) and 68% for TXB_2 (5.55 ng/g). Decreased prostaglandin production in corneas was associated with a 2.5-fold cellular increase in apoptosis and a 65% decrease in proliferation. Similar reductions in proliferation were observed in neovascular stroma (65–70%) of celecoxib-treated (dietary 160 ppm/day) xenograft tumors as well as in tumor cells (50–75%). Apoptosis was also increased in the tumor cells (2.2–3.0-fold) in response to celecoxib. Thus, the antitumor activity of celecoxib may be attributable, at least in part, to a direct effect on host stromal elements, such as the angiogenic vasculature.

Introduction

Epidemiological studies have shown a decreased risk of cancer in people who regularly take aspirin or other NSAIDs[2] (1). NSAIDs are known to be potent inhibitors of the COXs, a family of enzymes that catalyze the conversion of arachidonic acid to prostaglandins (2). Prostaglandins are locally acting molecules involved in both physiological and pathological functions (3). Prostaglandins derived from the constitutive COX-1 isozyme are produced in many tissues of the body and thought to be responsible for physiological activities including maintenance of the gastrointestinal mucosa, kidney, and platelet functions (4, 5). COX-2 is inducible by inflammatory stimuli, including cytokines, growth factors, and tumor promoters, and has been associated with inflammatory diseases and cancer (6). Most NSAIDs inhibit both COX-1 and COX-2. Whereas conventional NSAIDs are effective drugs for the treatment of pain and inflammation, with long-term use they have been known to inhibit the production of COX-1-derived prostaglandins needed for maintenance of gut, kidney, and platelet functions. These unwanted effects limit their use for some patients (7). Celecoxib and rofecoxib are relatively new drugs designed to treat the signs and symptoms of adult arthritis (8, 9). These drugs inhibit the inflammatory COX-2 enzyme at therapeutic doses in humans that do not lower gastrointestinal prostaglandin levels associated with mucosal protection and are thus not likely to cause the severe side effects associated with conventional NSAID use (10). In addition, celecoxib can be used to reduce the number of adenomatous colorectal polyps in patients with familial adenomatous polyposis, as an adjunct to usual care. Familial adenomatous polyposis is a genetic disease characterized by aberrant colon polyp formation that, if left untreated, results in a high incidence of colon cancer. Celecoxib effectively decreases the number and size of colon polyps with as little as 6 months of treatment (11). We are studying the role of COX-2 in cancer with the hypothesis that the historical ability of conventional NSAIDS to limit cancer growth is attributable to COX-2 inhibition, and that celecoxib will also inhibit cancer growth, but with increased safety at doses that do not inhibit COX-1.

Immunohistological studies have shown that COX-2 is expressed in a variety of solid human cancers in the malignant epithelial cells as well as the neovasculature that feeds the tumor (12). Normal vascular-associated cells express COX-1 but not COX-2. In the neovasculature of tumors, however, COX-2 appears to be induced, as determined by Western blot as well as immunohistology, whereas COX-1 levels are not different from normal vasculature. These findings suggest that inhibition of COX-2 may target the abnormal blood vessel growth associated with solid cancers that is necessary for tumor survival and metastasis. Therefore, inhibition of COX-2 without inhibition of COX-1 should spare normal vascular function, because COX-2 is not expressed in normal vasculature unassociated with pathological states such as cancer (12). Targeting tumor-associated, but not normal, vascular growth as a way of limiting tumor growth was proposed as an anticancer strategy by Folkman (13) as early as 1971. Since then, inhibition of angiogenesis has been shown to slow tumor growth in experimental models of cancer (14). Our previous studies using FGF-2-induced rat corneal angiogenesis have shown that this angiogenesis is COX-2 dependent and is suppressed dose dependently with oral administration of celecoxib. Celecoxib also inhibits tumor growth and metastasis of xenografts of human cancer cells in nude mice (12, 15, 16) as well as chemically (17–19) and genetically (20) induced rodent cancers.

The present studies examined the role of COX-2 in angiogenesis through the effect of celecoxib in two model systems, the FGF-2 rat corneal micropocket, and the human colon cancer cell xenografts, HT-29 and HCT116 cells, in athymic mice. We show that celecoxib inhibited the COX-2-derived prostaglandin production in the corneal micropocket and that these prostaglandins were associated with angiogenesis. In addition, celecoxib decreased proliferation and induced apoptosis of angiogenic cells in rat cornea. Similar antiproliferative effects of celecoxib were seen in murine models of tumor-derived angiogenesis, including antiproliferative effects of the tumor epithelia. Increases in tumor cell apoptosis with celecoxib treatment were also observed. Other groups have shown similar direct effects on cancer cells treated *in vitro* with celecoxib (21, 22), conventional NSAIDs (23), or other COX-2 inhibitors (22, 24, 25). However, these studies were done *in vitro*, and the effects were observed at high concentra-

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[2] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; FGF, fibroblast growth factor; BrdUrd, bromodeoxyuridine; PGE_2, prostaglandin E_2; TXA_2 and TXB_2, thromboxane A_2 and B_2, respectively; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

tions (50–100 μM) that may not be achievable in tumors *in vivo* and may be well beyond the doses required to inhibit prostaglandin production. Thus, these concentrations may not selectively inhibit either COX isoform (22, 26). Several hypotheses have arisen from these *in vitro* studies to explain a nonprostaglandin-dependent mechanism of apoptosis induction. One purpose of our studies was to investigate the effects of inhibiting the enzymatic activity of COX-2 on apoptosis and proliferation *in vivo*. Our results include an analysis of prostaglandins produced in the corneal micropocket model of angiogenesis at doses of celecoxib that do not inhibit COX-1, yet did induce apoptosis and inhibit proliferation. Thus, we provide evidence to support the notion that one of the main anticancer mechanisms of celecoxib is to decrease production of prostaglandins in the COX-2 expressing neovasculature of tumors, resulting in an inhibition of proliferation and induction of apoptosis in both the vasculature and the tumor.

Materials and Methods

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 basic FGF (FGF-2) or saline (placebo) was inserted into the pocket as described previously (16). Cyclooxygenase 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 either the day before surgery or 48 h after surgery, and continuing the length of the study. To assess the cellular proliferation in the corneas, the rats were injected with BrdUrd in saline at 0.1 mg/kg i.p. ~4 h before sacrifice. Four days after surgery, the corneas were examined under a slit lamp microscope, and the 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°), and applied to the formula: Area (mm^2) = ($CH/12$) × $3.14(r^2 - (r - VL)^2)$. While the rats were well anesthetized, blood for serum and plasma samples was taken by heart puncture, and either the whole eye or just the angiogenic portion (about one-third of the total corneal area) of both corneas was dissected. The rats were then immediately euthanized. All animal treatment protocols were reviewed by and were in compliance with Pharmacia's Institutional Animal Care and Use Committee.

Prostaglandin Extraction from Corneas. The angiogenic portions (about one-third of the total corneal area) of two corneas per anesthetized rat were dissected under a microscope, dipped briefly in 100 μM indomethacin in saline to stop prostaglandin synthesis and wash off excess blood, and placed in a Bio101 pulverizer tube H containing a ceramic pellet, garnet beads, and 0.5 ml of ethanol. The corneas were pulverized in a Bio101 FastPrep FP120 biopulverizer at setting 4 for 40 s. Protein precipitate was pelleted in a microcentrifuge, and the ethanol layer was removed to a clean tube. The ethanol was evaporated by vacuum centrifugation, the residue was redissolved in enzyme immunoassay (EIA) buffer (Cayman Chemical, Ann Arbor, MI), and samples were analyzed for PGE_2 and TXB_2 using an EIA kit (Cayman Chemical).

Human Colon Tumors in Nude Mice. Human colon carcinoma cells, HT-29 or HCT116, were implanted s.c. in the hind paws (1×10^6 cells) of nude (athymic) mice (n = 15/treatment group). Celecoxib therapy was initiated in the diet at 160 ppm (equivalent to 25 mg/kg/day p.o.) when tumors reached a mean volume of 100 mm^3 and maintained for the duration of the experiment. At the end of the experiment, the mice were sacrificed by CO_2 inhalation. The tumors were excised, sliced into 2-mm-thick sections, and fixed for immunohistology.

Immunohistochemical Analysis. Harvested eyes and tumors were fixed in Streck STF fixative for 12–18 h at 4°C. Corneas with iris intact were dissected from the eyes and bisected through the implanted pellet to expose cross-sections of the angiogenic region for microtomy. For BrdUrd and TUNEL analysis, the cornea halves and tumor slices were dehydrated in ethanol to xylene and embedded in paraffin. For immunohistochemistry, cornea halves and tumor slices were soaked in 15% sucrose in Tris-buffered saline for 6–7 h, followed by 30% sucrose in Tris-buffered saline for 24 h before freezing in OCT with liquid nitrogen-cooled isopentane.

Paraffin-embedded sections of eyes and tumors were cut to 4 μm thick, dewaxed, and rehydrated using routine procedures. For BrdUrd immunolocalization, sections were blocked for endogenous peroxidase (1% H_2O_2 in PBS for 10 min), treated with acid, and labeled with a biotin-conjugated mouse anti-BrdUrd antibody (Zymed Laboratories, South San Francisco, CA). For the TUNEL assay, sections were blocked for endogenous peroxidase, treated with 0.25% Triton X-100 in PBS at 50°C for 20 min, and incubated in terminal deoxytransferase enzyme with biotin dUTP and cobalt ion for 90 min at 37°C (Trevigen, Inc., Gaithersburg, MD). Anti-BrdUrd and TUNEL-labeled sections were visualized with streptavidin peroxidase and diaminobenzidine (Dako Corp, Carpinteria, CA), followed by hematoxylin counterstain to render proliferating or apoptotic nuclei dark brown and normal cell nuclei blue.

For COX immunolocalization, paraffin sections were antigen retrieved [0.1 M citrate (pH 5.6) at 95°C for 10 min] and incubated in specific anti-COX-1(Cayman 160109) or anti-COX-2 (Oxford PG27 or Cayman 160126) antibodies overnight at room temperature. COX antibody binding was detected with biotin goat antirabbit IgG, followed by streptavidin horseradish peroxidase and diaminobenzidine and hematoxylin counterstain.

For immunofluorescent staining, frozen sections of FGF-2-treated cornea and HT29 tumor sections were double labeled with endothelial cell-specific antibodies, anti-CD31 or MECA-32, and COX-2 antibody. Antirat CD31 (PharMingen, San Diego, CA) was used at 5 μg/ml, and biotin MECA-32 (PharMingen) was used at 2.5 μg/ml. Anti-COX-2 (Cayman 160126; Cayman Chemical Co.) was used at 1.25 μg/ml. All primary antibodies were incubated overnight at either 4°C or room temperature. For cornea sections, COX-2 antibody was visualized with biotin-conjugated goat antirabbit IgG (Jackson Immuno Research Labs) followed by Alexa 488-conjugated streptavidin. Anti-CD31 was with a rat-absorbed, goat antimouse IgG conjugate with Alexa 594. For tumor sections, MECA-32 was visualized with Alexa 488-conjugated streptavidin and COX-2 with swine antirabbit IgG (Dako Corp., Carpinteria, CA), followed by Alexa 594-conjugated streptavidin. All Alexa conjugates were purchased from Molecular Probes, Inc. (Eugene, OR). Stained sections were imaged on an Olympus AX-70 light microscope equipped with computer-controlled digital camera and imaging software.

Measurement of Proliferation and Apoptosis. Proliferation and apoptosis in cornea sections were measured by visually counting, with a ×40 objective, the labeled and unlabeled cells of microvessels in the angiogenic region of each cornea. For a given experiment, there were five animals/treatment group and three to four cornea pieces/animal. For each animal, the summed total cell count, 100–150, was divided by the sum total cells that were BrdUrd or TUNEL positive to obtain a proliferation or apoptotic index expressed as the percentage of total cells for that animal. The indexes for each treatment group were calculated as the average plus or minus the SE of the five animals in the group.

Proliferation and apoptosis were measured in two to three tumor sections from regions of tumor that contained healthy proliferating tissue typically found at the margins of the tumor. Necrotic tissue lying at the center of each tumor was excluded from analysis. Computer-based color image analysis was used with a method based on the differential absorption (27) to count total tumor cell nuclei and stained nuclei in image fields of tumor section. The proliferation and apoptosis indices were expressed as a percentage of total cells. All image fields were acquired with a ×20 objective and consisted of a 430 × 330 μm area of tumor section that typically contained 600-1000 nuclei for HT-29 and HCT116 tumors, respectively. For a given tumor, 10–15 images were analyzed, and the average proliferation or apoptotic index from these images represented the value for that animal. The individual tumor values were averaged for each treatment group and were expressed as the percent of total cells with SE.

Proliferation and apoptosis for endothelial cells was performed by visually counting the number of BrdUrd- or TUNEL-stained or unstained endothelial cell nuclei in microvessel structures in a ×40 high power field in two to three sections of tumor. For a given animal, 15–20 microvessels consisting of 100–150 endothelial cells were examined. The endothelial cell proliferation or apoptotic index for a given animal was obtained by dividing the total number of 3,3′-diaminobenzidine-stained nuclei by the total cell count. Indices from animals in the same treatment group were averaged and expressed as the mean with SE.

Results and Discussion

Basic FGF (FGF-2) corneal implants induce a robust angiogenic response in the normally avascular cornea characterized by endothelial-lined neovessels, sprouting from resident limbic vessels and growing toward the growth factor releasing pellet (Fig. 1*A*, *left* and *center*). Dosing FGF-2-implanted rats with 30 mg/kg/day of the COX-2 inhibitor celecoxib significantly inhibited neovascular growth by 78.6% [Fig. 1*A* (*center* and *right*) and Fig. 1*B*]. The antiangiogenic effect of celecoxib was substantial because measurement of the neovascular area does not account for the decrease in vessel density we observed with celecoxib treatment. In general, celecoxib-treated FGF-2-implanted corneas had vessels that were fewer in number, further apart, shorter in length, and covered less area than untreated FGF-2-implanted corneas (Fig. 1, *A* and *B*). We have shown previously that the angiogenesis produced by FGF-2 in this model is dose dependently decreased by COX-2 inhibition. Oral administration of an agent that selectively inhibits systemic COX-1 activity has no effect on FGF-driven angiogenesis. Thus, FGF-2-derived rat corneal angiogenesis depends on the activity of the COX-2, not the COX-1 isozyme (16).

To determine whether prostaglandins derived from COX-2 were produced in FGF-2-stimulated rat corneal angiogenesis, relevant portions of the corneas were harvested, and prostaglandins were extracted and assayed by ELISA. The FGF-2-releasing implants enhanced production of PGE_2 in the rat corneal stroma from 63.1 ng/g in unimplanted normal cornea to 338.6 ng/g in FGF-2-stimulated corneas (Fig. 1*C*). Similarly, TXB_2, the stable metabolite of active TXA_2, was increased from 2.0 ng/g in normal cornea to 17.53 ng/g in FGF-2 implanted cornea (Fig. 1*D*). Blocking COX-2 activity in FGF-2-implanted corneas with celecoxib (30 mg/kg/day) inhibited corneal neovascular area by 78.6% (4 days continuous exposure; Fig. 1B) and corneal PGE_2 and TXB_2 production by 78.5 and 68.3%, respectively (Fig. 1, *C* and *D*). Thus, inhibition of COX-2-derived prostaglandin production was closely associated with inhibition of angiogenesis (Fig. 1, *B–D*). Similar results were seen in a study using chemical cautery of rat corneas to induce angiogenesis. Corneal production of PGE_2 *ex vivo* is inhibited 75–80% by a COX-2 inhibitor (28). Thus, prostaglandins produced in angiogenic rat corneas are likely to be derived from COX-2, because this production can be decreased by inhibition of COX-2.

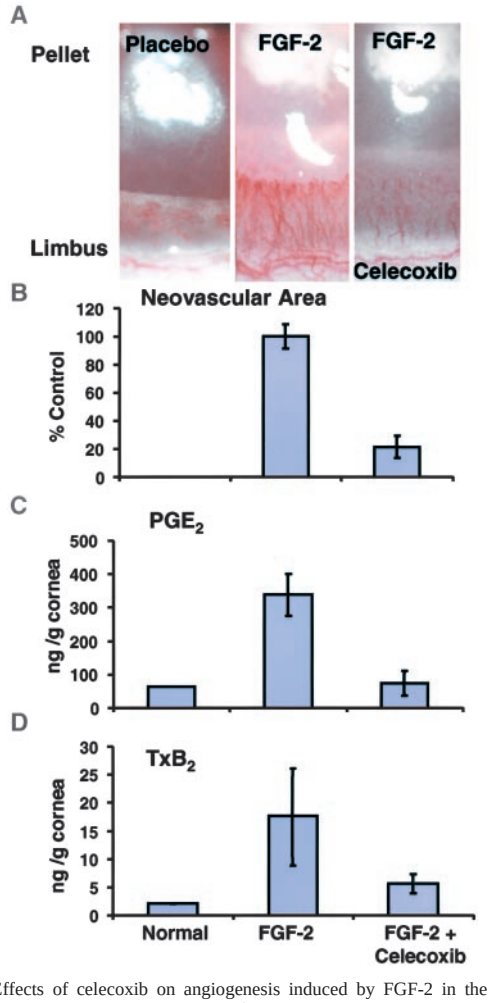

Fig. 1. Effects of celecoxib on angiogenesis induced by FGF-2 in the rat corneal micropocket model. *A*, slit lamp images of 4-day microvessel outgrowth from limbic vessels after implanting placebo pellet, FGF-2 releasing pellet only, and FGF-2 pellet in rats treated with celecoxib from time of implant. *B*, microvessel area measurements in normal, FGF-2 implanted, and FGF-2 implanted, celecoxib-treated corneas. PGE_2 (*C*) and TXB_2 (*D*) production in normal corneas and FGF-2-implanted corneas with and without celecoxib treatment is shown. *Bars*, SE.

The cellular expression of COX-2 protein in the angiogenic portion of corneas implanted with FGF-2 was examined by double immunofluorescent labeling using a specific COX-2 antibody (Fig. 2*A*) and an antibody directed against CD31 as a marker for endothelium (Fig. 2*B*, *red*). Nearly every cell that stained positively for CD31, an endothelial cell antigen (Fig. 2*B*, *red*), also stained positively for COX-2 by a level of 2–3-fold over background (Fig. 2*C*, *orange*). Other cells, not expressing CD31, were more intensely positive for COX-2 (Fig. 2*C*, *green*). These included macrophages, polynuclear cells, keratocytes, and other unidentified cells in the angiogenic area of the FGF-2-implanted corneas (Fig. 2*A*, *green*). On the basis of the intensity of label, COX-2 was expressed at a 5–6-fold higher level in macrophages than in CD-31-positive endothelial cells. Vascular cells of mature limbic blood vessels did not express COX-2 (data not shown). Placebo pellet implants containing no growth factor produced no angiogenesis (Fig. 1*A*), few infiltrating cells, and no significant COX-2 expression (data not shown). Thus, FGF-2 induced COX-2 protein expression in neovascular endothelial and other cells in the FGF-2-implanted angiogenic corneas (Fig. 2*A*; Ref. 16).

To further characterize the antiangiogenic effect of COX-2 inhibition, we compared endothelial cell proliferation (BrdUrd incorporation) and apoptosis (TUNEL assay) in FGF-2-implanted, celecoxib-treated corneas with vehicle-treated control corneas. In this study, celecoxib treatment (30 mg/kg/day) was initiated 36 h before sacrifice, on day 2.5 after FGF-2 implantation, to provide a sufficient number of sprouting vessels present in the corneas for counting and statistical analysis. BrdUrd incorporation was observed in endothelial cells and fibroblast-like cells and the basal layer of epithelial cells in the cornea in vehicle-treated controls (Fig. 2*D*). Celecoxib treatment inhibited endothelial cell proliferation by 2.5-fold as measured by a drop in proliferation index from 20 ± 1.5% in vehicle-treated controls to 7.3 ± 4% in celecoxib-treated corneas. No inhibitory effect of celecoxib treatment on corneal basal epithelial (Fig. 2*D*, *arrow*) or small bowel epithelial cell proliferation was observed (7.7 ± 0.3 BrdUrd-positive cells/crypt in vehicle-treated rats compared with 7.6 ± 0.3 cells/crypt for celecoxib-treated rats), indicating that celecoxib only inhibited the proliferation of COX-2-expressing cells and did not generate systemic antiproliferative effects. Celecoxib also had a profound effect on corneal neovascular endothelial cell homeostasis consisting of a 2.7-fold increase in apoptotic index from 3.0 ± 0.3% in vehicle to 8.1 ± 1.4% with celecoxib treatment (Fig. 2*E*). Thus, inhibition of COX-2 produced effects on the growth of the neovasculature at the cellular level consistent with antiangiogenic activity. Taken together, these data suggest that COX-2-derived prostaglandins play a major role in growth of new vascular-associated cells during angiogenesis *in vivo*. Thus, prostaglandin production resulting from

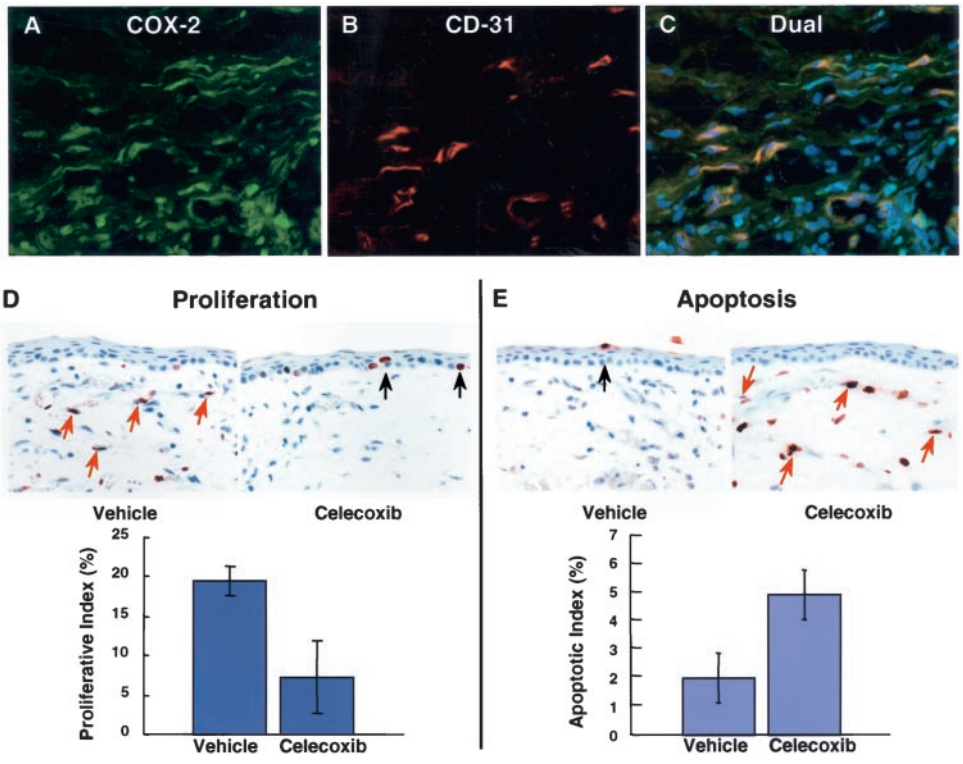

Fig. 2. COX-2 expression in FGF-2-implanted corneas and effects of celecoxib on endothelial cell proliferation and apoptosis. *A–C*, immunofluorescence localization of COX-2 (*green*; *A*) and endothelial cell marker, CD-31 (*red*; *B*) and colocalization (*C*) showing COX-2 labeling in cells expressing CD-31. The *orange*-colored cells in *C* are endothelial cells that are expressing COX-2. *Bars*, 50 μm. *D*, effect of celecoxib on endothelial cell proliferation as revealed by BrdUrd immunohistochemistry and labeling index in FGF-2 corneas. Vehicle-treated animals exhibited robust endothelial cell proliferation (*red arrows*) that was inhibited in celecoxib-treated animals. There was no observed effect of celecoxib treatment on homeostatic, corneal epithelial cell proliferation (*black arrows*). Endothelial cell proliferation, determined by the ratio of BrdUrd-positive microvessel cells to total microvessel cells with celecoxib treatment was significantly ($P < 0.04$) inhibited compared with vehicle treatment of animals. *E*, effect of celecoxib on endothelial cell apoptosis as revealed by TUNEL histochemistry. FGF-2 corneas in vehicle-treated animals contained a few apoptotic cells, mostly neutrophils. Apoptotic epithelial cells in the granulosum layer undergoing normal epithelial maturation (*black arrow*) were also observed and served as an internal control for the TUNEL assay. With celecoxib treatment, apoptotic cells associated with microvessels were more abundant (*red arrows*). The apoptosis index, determined by the ratio of TUNEL-positive endothelial cells and total endothelial cells, was significantly ($P < 0.009$) elevated with celecoxib treatment compared with vehicle treatment. *Bars*, SE.

cytokine induction of COX-2 is proangiogenic and was blocked by COX-2 inhibition *in vivo*. Suppressed levels of COX-2-derived prostaglandins were closely associated with decreased angiogenic activity as indicated by less neovascular area, slower proliferation, and increased apoptosis.

To demonstrate that the antiangiogenic effects of celecoxib observed in the corneal angiogenesis model may also be responsible for some of the antitumor activity of celecoxib *in vivo*, celecoxib treatment was evaluated in murine hosts using xenografts of human colon cancer cells. COX-2 is expressed *in vitro* in some human colorectal adenoma cell lines (23); however, when implanted s.c. into immune compromised mice, some human cancer cells seem to lose the ability to express COX-2. The human colon cancer cell line HT29 expresses both COX-1 and COX-2 enzymes *in vitro* by Western blot analysis (not shown). Another human colon cancer cell line HCT116 expresses neither enzyme *in vitro*. Interestingly, when either of these human cancer cells is injected into the footpad of nude mice, the resulting tumors' epithelial cells do not express human COX-2 (Fig. 3*A*, *blue*). Immunohistology confirmed COX-1 expression but not COX-2 expression in HCT-116 tumor cells (Fig. 3, *B*, *C*, and *D*) and revealed the expression of COX-2 only in the murine vascular-associated cells in the tumor stroma (Fig. 3*A*, *yellow*, and *D* and *E*). Similar results were found in HT-29 tumors (not shown). Thus, in both xenografts, COX-2 expression is limited to vascular regions of the tumors and is not supplemented with COX-2 expression from the tumor cells, unlike human carcinomas that do express COX-2 in some epithelial cells as well as neovasculature. It is therefore reasonable to suppose that inhibition of COX-2 activity in these experimental tumors should primarily affect the cells contained in and adjacent to neovascular areas.

Despite the lack of COX-2 in the tumor cells, celecoxib inhibited both HT-29 and HCT-116 xenograft tumor growth by 75 and 74%, respectively, after 40 days in this and our previous work with HT29 (16). Consistent with growth inhibition, sections from tumors grown in mice treated with celecoxib displayed a marked reduction in BrdUrd-positive cells compared with vehicle-treated control (Fig. 4*A*). Proliferation indices in tumor cells were reduced from 21.6 $\pm$ 3.2% and 15.4 $\pm$ 1.3 in vehicle-treated to 10.8 $\pm$ 1.0 and 3.6 $\pm$ 0.5% in celecoxib-treated HT-29 and HCT-116 tumors, respectively (Fig. 4*B*). In addition to inhibiting proliferation, celecoxib consistently induced apoptosis in the tumor cells compared with vehicle-treated controls by a factor of 2.2–3.0-fold (Fig. 4, *D* and *E*). Because the tumor epithelial cells were not expressing COX-2 and the murine neovascular cells did, tumor cell effects may have been a result of slowing the vascular growth. Indeed, BrdUrd analysis of the microvessel endothelial cell population in these sections showed that celecoxib profoundly inhibited proliferation (Fig. 4*C*), consistent with the FGF-2-induced corneal micropocket model. Vascular cell proliferation indices for HT-29 and HCT116 tumors dropped from 16.8 and 9.3% in vehicle-treated controls to 6.1 and 3.2%, respectively, in celecoxib-treated tumors (Fig. 4*C*). In addition, a modest increase in apoptosis in the stromal cells of HT29 tumors was observed, from 7 $\pm$ 0.7% in vehicle-treated tumor stroma to 11.8 $\pm$ 1.1% in celecoxib-treated tumor stroma (not shown). Thus, effects on neovascular cells may be responsible for the antitumor effects observed.

Neovasculature-associated cells are important components of the cells comprising established tumors and include endothelial cells, pericytes, and inflammatory cells. Immunohistological staining of human colon cancer archival tumors shows that COX-2 enzyme is expressed in the vascular-associated cells as well as in some cells of the neoplastic epithelium (6, 12, 16). In normal, noncancerous intestine, COX-2 is detected by immunohistological staining only at low levels in the proliferating cells at the base of the crypts and not in the cells comprising the normal vasculature. At every stage of hyperproliferation leading to cancer, however, COX-2 is overexpressed in the neoplastic epithelium and is also found in the neovascular endothelium (16). In contrast, COX-1 is expressed in normal epithelial cells of the gut and normal vascular cells as well as cancerous epithelial cells at all stages, making it difficult to determine whether COX-1 plays a role in cancer development (12). Thus, one of the ways in

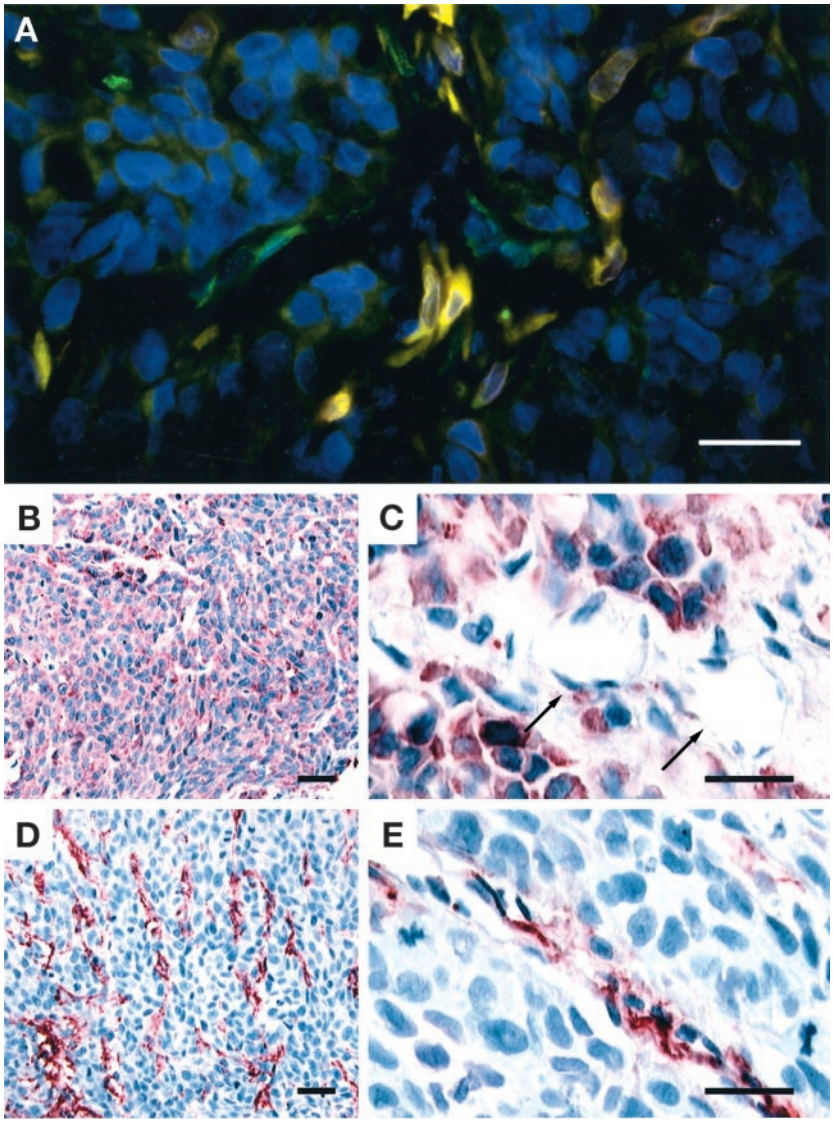

Fig. 3. COX-2 expression in HT29 and HCT116 human colon cancer xenograph tumors is restricted to endothelial and stromal cells. *A*, in the HT29 tumor model, double label immunofluorescence reveals that COX-2 (*green*) colocalizes with endothelial cell marker MECA-32 (*yellow* cells) in some cells. Unidentified stromal cells (*green*) also express COX-2. *B* and *C*, COX-1 immunohistochemistry in HCT116 tumor model demonstrated that COX-1 is highly expressed in the tumor cells (*B*) but is not present in putative endothelial or stromal cells (*C, arrows*). *D* and *E*, COX-2 expression in HCT116 is almost exclusively localized in the endothelial and stromal cells. *Bar*, 50 μm.

which malignant colon cells differ from normal colon cells is the increased expression of COX-2 in cancer. Interestingly, another difference is the contrast of normal vascular expression of COX-1 to the neovascular expression of COX-2 in intestinal carcinomas, as well as other epithelial cancers (12).

COX-2 activity produces prostaglandins that can have both autocrine and paracrine effects on proliferation and migration of endothelial cells *in vitro*. Experiments show that cytokines derived from nonendothelial cells involved in angiogenesis stimulate the induction of COX-2 in vascular endothelial cells. In one study, oncostatin M, a cytokine of the interleukin-6 family secreted by macrophages, has a potent proliferative effect associated with induction of COX-2 in

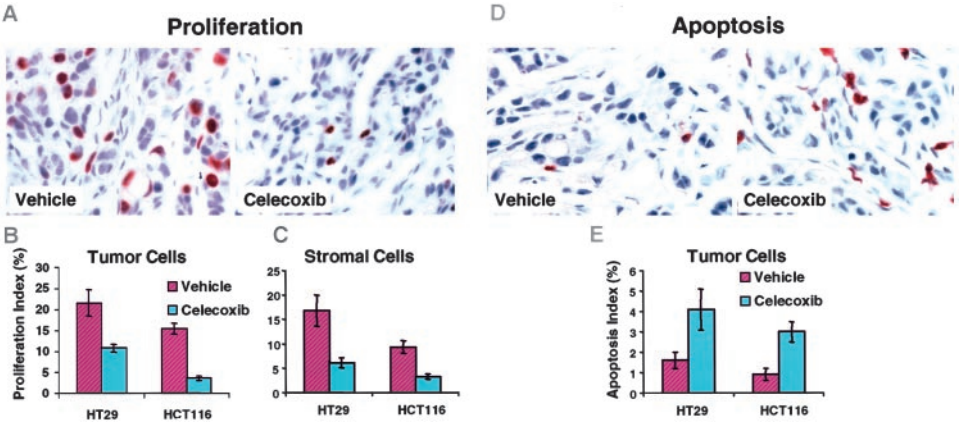

Fig. 4. Effect of celecoxib treatment on proliferation and apoptosis in HT29 and HCT116 tumor models. *A*, BrdUrd immunohistochemistry of vehicle- and celecoxib-treated tumors illustrated the profound inhibitory effect of celecoxib on tumor and stromal cell proliferation. *B*, in both models, celecoxib inhibited proliferation of tumor cells by 50–60%. *C*, stromal cell proliferation was also inhibited in both models by a similar amount. *D* and *E*, TUNEL assay comparison of vehicle- and celecoxib-treated tumors revealed a marked induction of apoptosis in tumor cells in both models. The apoptosis index of 1–1.8% in vehicle-treated tumors rose to 3–4% in celecoxib-treated tumors. *Bars*, SE.

angiogenic human microvascular endothelial cells. COX-2 inhibition limits the proliferative effect of oncostatin M (29). Thus, COX-2 can be directly induced in endothelial cells in response to cytokines and plays a proliferative role. The promigratory effects of prostaglandins on vascular endothelial cells *in vitro* have also been explored. Adding authentic PGE_2 to endothelial cells mimics the migration-stimulatory activity of head and neck squamous cell carcinoma cell supernatants (30). TXA_2 is one of several eicosanoid products generated by activated human endothelial cells. Angiogenic basic FGF-2 or vascular endothelial growth factor increases TXA_2 synthesis in endothelial cells *in vitro* 3–5-fold. These growth factors induce endothelial cell migration as well. A TXA_2 mimetic, U46619, stimulates endothelial cell migration, whereas either inhibition of TXA_2 synthesis or blockade of TXA_2 receptor reduces growth factor-driven endothelial cell migration (31). Thus, cytokine stimulation of endothelial cells, such as those found in the FGF-2-implanted corneas, may induce COX-2, thereby increasing production of COX-2-derived prostaglandins and prostaglandin-mediated proliferation. We propose that whether the source of COX-2 in the cornea is the endothelial cell or some other cell type, one effect of COX-2-derived prostaglandins on endothelial cells is to promote angiogenesis.

Prostaglandins by themselves can generate an angiogenic response in some *in vivo* models. In a chicken embryo study, prostaglandin E_2, in amounts equivalent to those seen in tumor nodules can elicit an angiogenic response to slow-release pellets placed on the chorioallantoic membrane of chicken embryos (32). In the rabbit cornea, PGE_1 also induces neovascularization and acts as an angiogenesis factor (33). Prostaglandins E_2 and E_1 can induce an intense capillary sprouting from rat femoral vein when administered into the soft connective perivascular tissue (34). A TXA_2 agonist, U46619, reconstitutes an angiogenic response under COX-2-inhibited conditions in FGF-2-induced corneal angiogenesis (35). Thus, our data and these reports demonstrate that E-series prostaglandins and thromboxanes produced by COX-2 can function as inducers of angiogenesis (32, 35).

Neovascular cells associated with tumors consistently express COX-2, regardless of the COX profile of the tumor cells (12). Our current data indicate, in both cornea and tumor angiogenic models, that COX-2 is expressed in vascular endothelial cells, and that inhibition of its enzymatic activity by celecoxib inhibits angiogenesis. Thus, we hypothesized that we can inhibit growth of tumors *in vivo* by inhibition of the growth of COX-2-dependent vascular cells recruited by the tumor for its survival. Indeed, our data from HT-29 and HCT116 tumor models clearly suggest that significant inhibition of tumor growth may be achieved by COX-2 inhibition, even when malignant epithelial cells do not express COX-2. Many human cancers do express COX-2 in some of the tumor epithelia; however, these data suggest that COX-2 inhibition in angiogenic cells may be enough to slow tumor growth, regardless of the COX-2 status of the tumor. Inhibition of COX-2 lowered prostaglandin levels in the corneal model of FGF-2-driven angiogenesis in a correlative manner to inhibition of angiogenesis. Thus, we propose that, in tumors, similar angiogenesis is dependent on COX-2-derived prostaglandins, and that lowering the levels of these enzymatic products may be enough to slow the growth, by inhibiting proliferation and induction of apoptosis, of neovasculature, and thus tumor growth. These studies support the possibility that COX-2 inhibitors such as celecoxib may have anticancer effects in humans. Clinical trials will be required to determine whether cancer patients will benefit from treatment with celecoxib.

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