Inhibition of Cyclooxygenase-2 by Rofecoxib Attenuates the Growth and Metastatic Potential of Colorectal Carcinoma in Mice

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ABSTRACT

A large number of epidemiological studies have shown that regular use of aspirin and other nonsteroidal anti-inflammatory drugs (NSAIDs) results in a 40–50% reduced risk of colorectal cancer (CRC). Furthermore, NSAIDs cause the regression of preexisting adenomas in patients with familial adenomatous polyposis and significantly inhibit tumor growth in animal models of CRC. To establish a CRC liver metastasis model, we implanted mouse colon tumor MC-26 cells into the splenic subcapsule of BALB/c mice, after which mice were given either standard chow or chow containing the cyclooxygenase (COX)-2-specific inhibitor rofecoxib, alone or in combination with the standard antineoplastic agents, 5-fluorouracil or irinotecan. After 14 days, mice that were given rofecoxib or irinotecan, but not 5-fluorouracil, had significantly smaller primary tumors and fewer metastases. Rofecoxib, at clinical anti-inflammatory plasma concentrations, enhanced the effects of both antineoplastic agents when used in combination. Biochemical analyses of the primary splenic tumor in rofecoxib-treated mice showed no alteration in COX-1 expression, but significant decreases in the expression of the tumor-promoting proteins COX-2, cyclin D1, cytosolic β-catenin, matrix metalloproteinases and -9, and vascular endothelial cell-derived growth factor. Rofecoxib also decreased growth-enhancing prostaglandin E2 and tumor-suppressive interleukin-10, whereas antineoplastic interleukin-12 was increased. Two separate survival studies were performed. When mice were fed chow containing 0.01% rofecoxib beginning on day 0 after tumor cell implantation, which achieved clinical anti-inflammatory plasma concentrations, survival time was significantly longer compared with mice given control chow. After 30 days, mortality in the control group was 90%, whereas only one mouse (5%) treated with rofecoxib had died after 30 days. In the second survival study, all of the mice were initially fed with regular chow after tumor cell implantation. On day 7, mice were randomly divided into three dietary groups: control chow, low-dose (0.01%) rofecoxib chow, and high-dose (0.025%) rofecoxib chow. After 28 days, mortality was 100%, 20%, and 10% in control, low-, and high-dose rofecoxib fed groups, respectively. These studies demonstrate that rofecoxib decreases the growth and metastatic potential of CRC in mice through multiple mechanisms. These studies in mice also provide important information that supports the benefit of COX-2 inhibition, not only in the prevention of CRC, but also potentially in the treatment of this common malignancy. Clinical trials will be necessary to assess the efficacy of COX-2 inhibitors as adjuvant therapy for early-stage disease and as potential agents, either alone or in combination, with more established drugs, for the treatment of refractory CRC.

INTRODUCTION

CRC is second only to lung cancer as a cause of death from malignant disease in the United States (1), with one-half of patients incurable at their presentation (2). Nearly 130,000 new cases of CRC were diagnosed in the United States in 1999, resulting in 56,600 associated deaths (3). Approximately 6% of Americans will develop CRC during their lifetime, and 2.6% will die from this disease (4). Chemotherapeutic modalities to treat refractory CRC, although associated with significant toxicity, have provided only minimal benefit in improving survival (5, 6). A large number of epidemiological studies have shown that regular use of aspirin or other NSAIDs results in a 40–50% reduced risk of CRC (7, 8). Furthermore, NSAIDs cause the regression of preexisting adenomas in patients with familial adenomatous polyposis (9) and significantly inhibit tumor growth in animal models of CRC (8, 10).

The mechanism of inhibition of COX activity by aspirin and NSAIDs was first described by Vane in 1971 (11). This observation led to the hypothesis that both the toxicity and efficacy of NSAIDs are mediated through the inhibition of COX-mediated prostaglandin synthesis (12). In the early 1990s, several groups reported the discovery of two COX isoforms, a constitutively expressed isoform, COX-1, and a strongly inducible form, COX-2, which is involved in growth proliferation and the inflammatory response (13, 14). Despite ~60% amino acid identity, the two COX isoenzymes are encoded by distinct genes and differ significantly with regard to tissue-specific distribution (12). COX-1 appears to function as a physiological regulatory enzyme in most tissues, including the gastric mucosa, the kidney, and platelets, whereas COX-2 is nearly undetectable in most (but not all) tissues under normal physiological conditions (12). COX-2 mRNA expression and protein was found to be enhanced in human colorectal adenomas and adenocarcinomas, leading to the hypothesis that selective COX-2 inhibition might thereby reduce the development of CRC (15–17). Specific COX-2 inhibition, either by targeted knockout of the COX-2 gene or by pharmacological intervention, has been shown to effectively decrease the growth of murine intestinal adenomas (17–19). In a rat model of chemical-induced CRC, the COX-2 selective inhibitor celecoxib suppressed the formation of azoxymethane-induced aberrant crypt foci (20). Celecoxib also inhibited the incidence and multiplicity of colon tumors by ~93 and 97%, respectively, and diminished the overall rat colon tumor burden by ~87% (21). A recent human study demonstrated that selective COX-2 inhibition with celecoxib in 77 individuals with familial adenomatous polyposis decreased the mean number of colonic polyps and the polypl burden by 28.0 and 30.7%, respectively (9).

No published studies have reported the benefits of specific COX-2 inhibitors in treating metastatic cancer. However, one clinical study involving the use of the potent nonselective COX inhibitor indomethacin demonstrated that the treatment of end-stage metastatic patients (mainly CRC and liver, pancreas, and gastric primary cancers) resulted in increased survival (250–510 days), less pain, and diminished use of other analgesics (22, 23).

In the present study, we sought to determine whether rofecoxib, a specific inhibitor of COX-2, could reduce primary tumor growth and liver metastatic potential of mouse colon tumors in vivo. We used BALB/c mice, in which MC-26 cells, a transplantable mouse CRC...
cell line expressing COX-2 protein, were implanted surgically into the splenic capsule. Mice were then administered either standard chow or chow containing the COX-2-specific inhibitor rofecoxib alone, or in combination with standard antineoplastic agents. Our initial hypothesis was that rofecoxib alone would have minimal effect but would have additive or synergistic effects with the standard antineoplastic agents. Our studies demonstrate that COX-2 inhibition by rofecoxib alone decreases the growth and liver metastatic potential of CRC in mice, in addition to augmenting the antineoplastic properties of the standard chemotherapeutic agents.

MATERIALS AND METHODS

Preparation of Rofecoxib and Other Antineoplastic Agents

We purchased 5-FU and LV from Sigma Chemical Co. (St. Louis, MO). The working solution was prepared by adding 5-FU and LV to 0.9% NaCl adjusted to pH 8.6 with 0.01 M NaOH, after which the solution was sonicated for 5 min and then filtered through a 0.22 μm filter. We obtained CPT-11 from Pharmacia (Kalamazoo, MI) and prepared the working solution by diluting 20 mg/ml of the agent into a 2 mg/ml concentration with 5% glucose. 5-FU, LV, and CPT-11 were injected i.p. at doses of 30 mg/kg, 50 mg/kg, and 30 mg/kg, respectively. We prepared 0.01% rofecoxib chow, equivalent to a dose of 20 mg/kg/day, for use in these experiments.

Cell Line

We obtained MC-26 cells, a transplantable mouse colon cancer cell line (24), from Dr. K. K. Tanabe Massachusetts General Hospital, (Boston, MA) and maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD), supplemented with 10% FBS and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin), at 37°C in a humidified atmosphere of 95% air/5% CO₂.

Animal Preparation, Treatment, and Surveillance

We purchased 6 to 10-week-old male BALB/c mice from Taconic (Germantown, NY). We harvested MC-26 cells from subconfluent cultures using trypsin-EDTA (Life Technologies, Inc.), followed by centrifugation at 300 × g for 15 min at room temperature. We then resuspended cells in serum-free DMEM or HBSS (Life Technologies, Inc.), and the cell number was adjusted to a final concentration of 200,000 cells/ml. A 1-cm incision was made in the mice under i.p. anesthesia with pentobarbital (Abbott Laboratories, North Chicago, IL) at 65 mg/kg body weight. Using a 27-gauge needle and a 1-ml syringe, we injected 100 μl of tumor cell suspension (2 × 10⁶) into the subsplenic capsule of mice. All of the animal studies were conducted using a protocol approved by the Institutional Animal Care and Use Committee at Boston University Medical Center.

On day 0, after complete recovery from surgery, we randomly divided mice into eight groups: Group 1, control (n = 20), received control chow plus 0.9% NaCl or 5% glucose i.p. injection on days 4, 5, 7, 8, 11, and 12; Group 2, rofecoxib alone (n = 20), received rofecoxib 0.01% chow, and 0.9% NaCl or 5% glucose i.p. injection on days 4, 5, 7, 8, 11, and 12; Group 3, 5-FU/LV alone (n = 11), received control chow plus 5-FU 30 mg/kg and LV 50 mg/kg on days 5, 7, and 12. Injection of 5-FU was performed 1 h after LV injection. Five % glucose i.p. injection was given on days 4, 8, and 11; Group 4, CPT-11 alone (n = 11), received control chow plus CPT-11 30 mg/kg on days 4, 8, and 11, and 0.9% NaCl i.p. injection on days 5, 7, and 12; Group 5, rofecoxib/5-FU/LV (n = 11), received rofecoxib chow plus 5-FU 30 mg/kg and LV 50 mg/kg on days 5, 7, 12, and 5% glucose i.p. injection on days 4, 8, and 11. Group 6, rofecoxib/CPT-11 (n = 11), received rofecoxib chow plus CPT-11 30 mg/kg on days 4, 8, and 11, and 0.9% NaCl i.p. injection on days 5, 7, and 12; Group 7, 5-FU/LV/CPT-11 (n = 11), received control chow plus 5-FU 30 mg/kg and LV 50 mg/kg on days 5, 7, and 12, and CPT-11 30 mg/kg on days 4, 8, and 11; Group 8, rofecoxib/5-FU/LV/CPT-11 (n = 11); received rofecoxib chow plus 5-FU 30 mg/kg and LV 50 mg/kg on days 5, 7, and 12, and CPT-11 30 mg/kg on days 4, 8, and 11.

On day 14, blood samples were obtained, after which all of the mice were sacrificed. During the dissection, primary (spleenic) tumor size was determined by weighing and then measuring the longest and shortest diameters of the tumor. Tumor volume (mm³) was calculated using the standard formula: tumor volume = (shortest diameter)² × (longest diameter) × 0.5. We fixed a portion of the tumor tissue in 10% formalin for subsequent histological examination and then flash-froze the remaining tissue in liquid nitrogen and stored it at -70°C. Metastatic nodules in the liver were detected macroscopically and confirmed microscopically, and the percentage of mice with hepatic metastasis was recorded.

In a separate experiment, to identify the effect of rofecoxib on liver weight, five mice without tumor cell infection were fed 0.01% rofecoxib chow, and another five mice without tumor cell infection were fed control chow for 14 days. All of the mice in both groups were sacrificed on day 14, and liver weights were measured.

Survival Studies

Two separate experiments were performed to assess survival.

Protocol 1. On day 0, after complete recovery from surgery, mice were randomly divided into two groups (n = 20 in each group): a control group, in which mice were fed with regular chow; and a rofecoxib group, in which mice were fed with rofecoxib 0.01% chow (~20 mg/kg mouse weight, which achieved a plasma concentration of 0.26 μg/ml); and a high-dose rofecoxib group, which received chow containing rofecoxib 0.025% (~50 mg/kg mouse weight, which achieved a plasma concentration of 0.55 μg/ml). The survival study was terminated on day 30.

Protocol 2. On day 0, after complete recovery from surgery, all of the mice were fed regular chow. On day 7, mice were randomly divided into three groups (n = 20 in each group): a control group, which received regular chow; a low-dose rofecoxib group, which received chow containing rofecoxib 0.01% (which achieved a plasma concentration of 0.26 μg/ml); and a high-dose rofecoxib group, which received chow containing rofecoxib 0.025% (~50 mg/kg mouse weight, which achieved a plasma concentration of 0.55 μg/ml). The survival study was terminated when mortality in the control group reached 100%.

Preparation of Microsomal Membranes from Splenic Tumor Tissue

Splenic tumor tissue was excised, immediately frozen in liquid N₂, and stored at -70°C. Frozen tissues were thawed in ice-cold homogenization buffer [50 mM potassium phosphate (pH 7.1), containing 0.1 M NaCl, 2 mM EDTA, 0.4 mM phenylmethylsulfonyl fluoride, 60 mM soybean trypsin inhibitor, 2 g/ml leupeptin, 2 g/ml aprotinin, and 2 g/ml pepstatin, all from Sigma Chemical Co.]. Tissues were disrupted twice, for 10 s each, on ice using a tissue homogenizer (IKA Labortechnik, Staufen, Germany). Samples were homogenized by sonication at 4°C using a Cole Parmer 4710 series ultrasonic homogenizer (Cole Parmer Instrument Co., Chicago, IL). Debris was removed by centrifugation at 1,000 × g for 15 min at 4°C, and the resultant supernatants were subjected to centrifugation at 100,000 × g for 30 min at 4°C. Membrane fractions were resuspended in homogenization buffer and then sonicated to obtain a homogeneous membrane suspension. Protein concentrations were determined for each sample using a protein assay kit (Bio-Rad, Mississauga, Ontario, Canada), and equal gel loading was determined by Coomassie Blue staining (Sigma).

SDS-PAGE and Immunoblot Analysis

Microsomal and cytosolic fractions were mixed with 0.5 volume of SDS sample buffer [20 mM Tris-HCl (pH 6.8), containing 0.4% (w/v) SDS, 4% glycerol, 0.24 M β-mercaptoethanol, and 0.5% bromphenol blue], boiled for 5 min and analyzed by SDS-PAGE on 9 × 10 cm precast 4–20% Tris-glycine acrylamide gels (NOVEX, San Diego, CA), according to the method of Laemmli (25). Proteins were electroethoretically transferred to nitrocellulose membranes, as described previously (26). Primary antibodies were to COX-1 (MF24; Merck-Frosst, Montreal, Canada), COX-2 (MF243; Merck-Frosst), β-catenin (Sigma), cyclin D1 (BD Transduction), MMP-2 (Oncogene), MMP-9 (Oncogene), and VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) were used at final dilutions of 1:3,000; 1:7,500; 1:5,000; 1:1,000; 1:1,000; 1:200, respectively, according to the manufacturers’ instructions. The second-ary horseradish peroxidase-linked goat anti-rabbit or goat antinouse IgG antibodies (Santa Cruz) were used at dilutions of 1:2,000 to 1:6,000. Immunodetection was performed using enhanced chemiluminescence according to the manufacturer’s instructions (Amersham, Elk Grove Village, IL). Protein bands were visualized using a Fuji LAS-1000 plus Luminescent Image Analyzer.
ELISA and Measurement of Plasma Rofecoxib Levels

The concentrations of IL-10, IL-12, and PGE2 in splenic tumor cytosolic fractions were determined by quantitative ELISA, according to the manufacturer’s directions. IL-10 and IL-12 immunoassay kits were purchased from Biosource International (Camillaro, CA), and PGE2 kits were purchased from Assay Designs, Inc. (Ann Arbor, MI).

Plasma rofecoxib levels were measured by HPLC. A set of four rofecoxib standards, ranging from 0.1 to 5.0 μg/ml (R² > 0.9999), were prepared in acetonitrile. We added 100 μl of blank mouse plasma to each 100-μl aliquot of the standards and then mixed the standards on an automatic vortex mixer for 10 min. The sample was centrifuged at 14,000 rpm for 15 min, and the supernatant was transferred to a HPLC vial for injection. Sample preparation was identical to the standard preparation procedure with the exception of a 100-μl aliquot of acetonitrile added to 100 μl of plasma sample. An HP1090 system equipped with an UV detector was fitted with an Eclipse XDB-C18 Rapid 74Resolution (4.6 × 75 mm, 3.5 μm) analytical column. We used a flow rate of 1.0 ml/min with a solvent ratio of 65% aqueous (0.1% trifluoracetic acid in water) to 35% organic (0.1% trifluoracetic acid in acetonitrile), an oven temperature of 40°C, and an injection volume of 25 μl. Rofecoxib was detected at a wavelength of 275 nm.

Statistical Analysis

Using SAS 8.0, we performed one-way ANOVA to compare tumor volume and liver weight and densitometric values of Western blot bands among the different animal groups, followed by Tukey’s procedure for paired comparisons. We used the Fisher Exact χ² test for categorical data such as the incidence rate of liver metastasis. We performed survival analysis using the Kaplan-Meier method and log-rank tests. We assigned statistical significance if P < 0.05.

RESULTS

Plasma Rofecoxib Concentrations. Rofecoxib could be detected in the plasma of all of the groups treated with rofecoxib alone or in combination with standard antineoplastic agents. Plasma drug concentrations in mice fed with 0.01% rofecoxib chow were similar to trough levels achieved at steady-state with once daily 25-mg dosing in a 70-kg human (27), and no significant differences were detected among the various groups (Fig. 1).

Effect of Rofecoxib Treatment on the Expression of COX-1, COX-2, Cyclin D1, β-Catenin, MMP-2, MMP-9, and VEGF. We examined the effect of rofecoxib treatment on the expression of mouse splenic tumor proteins involved in proliferation, cell cycle regulation, angiogenesis, and metastasis. Splenic tumor samples from rofecoxib-treated or control groups were assessed for expression of COX-1, COX-2, β-catenin, cyclin D1, MMP-2, MMP-9, and VEGF proteins by specific immunoblot analyses. Mice treated with rofecoxib had a statistically significant reduction in splenic tumor levels of COX-2, cyclin D1, β-catenin, MMP-2, and VEGF protein, in comparison with mice treated with control chow (Fig. 2, B–E, G). Mice treated with rofecoxib showed a trend toward reduction of MMP-9 protein expression; however, this reduction did not reach statistical significance (Fig. 2F).

Effect of Rofecoxib Treatment on the Expression of PGE2, IL-10, and IL-12. Splenic tumor samples from rofecoxib-treated and control mice were assessed for PGE2, IL-10, and IL-12 concentrations by quantitative ELISA, as described above. The concentration of growth-proliferative, immunosuppressive PGE2 was reduced in the splenic tumors of mice treated with rofecoxib in comparison with control animals (Fig. 3A). This reduction in PGE2 is consistent with the reduction in the COX-2 enzyme in the tumors of rofecoxib-treated animals (Fig. 2B). The tumor promoting IL-10 cytokine was significantly reduced in tumor samples of rofecoxib-treated animals as compared with controls (Fig. 3B). In contrast, levels of the potent proinflammatory tumor suppressive cytokine IL-12 were markedly increased in rofecoxib-treated splenic tumors as compared with control mouse splenic tumors (Fig. 3C).

Effect of Rofecoxib Treatment on Tumor Growth and Metastasis and on the Survival of Mice with Implanted Splenic CRC Cells. Rofecoxib alone, or in combination with standard antineoplastic agents, significantly inhibited in vivo colon cancer growth and the incidence of spontaneous liver metastasis (Fig. 4). Primary splenic tumor volume was significantly decreased in all of the groups, although to a lesser extent in those mice treated with 5-FU/LV (Fig. 5A). Moreover, rofecoxib potentiated the effects of both 5-FU/LV and CPT-11 on tumor volume (Fig. 5A). Liver weight (Fig. 5B) and the incidence of liver metastasis (Fig. 5C) were not significantly affected in mice treated with 5-FU/LV alone. In contrast, both rofecoxib and CPT-11 decreased liver weight and metastasis significantly, and the effects of rofecoxib and CPT-11 were additive (Figs. 5, 6B, and 6C). We did not detect metastases in any of mice treated with both rofecoxib and CPT-11 (Fig. 5C). Liver weight and metastasis were diminished in mice treated with a combination of rofecoxib and 5-FU/LV compared with the latter alone but were increased compared with mice treated with rofecoxib alone.

In the initial survival study, in which mice were fed with 0.01% rofecoxib chow on day 0, the survival time in mice treated with rofecoxib chow was significantly longer than in mice given control chow. After 30 days, mortality in the control group was 90%, whereas only one mouse (5%) treated with rofecoxib had died after 30 days (P < 0.00001; Fig. 6A). In the second survival study, mice were fed with regular chow on recovery from surgery, and on day 7, they were randomly divided into three groups. All of the mice fed with control chow died by day 28, and mortality rates were 100, 20, and 10%, respectively, in the control, 0.01 and 0.025% rofecoxib groups (P < 0.0001 for both treatment groups; Fig. 6B).
DISCUSSION

A large body of experimental evidence supports the premise that COX-2 expression may contribute to tumorigenesis and that COX-2 inhibitors might be useful in the prevention of intestinal polyposis and colon cancer (9, 22, 23). Increased levels of COX-2 mRNA and COX-2 protein have been found in colonic tumors and polyps, in comparison with normal colonic epithelium (15–17). Several mechanisms have been supported for why COX-2 expression in neoplastic tissues enhances tumor growth. There is evidence for amplification of tumor cell proliferation by COX-2-produced PGE2 and inhibition of tumor cell apoptosis, enhancement of stromal cell angiogenesis, and decreased immune surveillance of tumor cells. Recently, Dormond et al. (28) demonstrated an important functional link among COX-2, integrin αVβ3, and the small GTPases Cdc42 and Rac. Using human umbilical vein endothelial cells, they found that both NS-398 and indomethacin, but not the specific COX-1 inhibitor SC-560, suppressed the activity of integrin αVβ3 (an adhesion receptor critically involved in mediating tumor angiogenesis) and prevented activation of Cdc42 and Rac, which are critically involved in regulating cell migration after integrin engagement (28).

COX-2-produced prostaglandins also increase the metastatic potential of human CRC cells by enhancing proteolysis of the basement membrane (15). Tsujii et al. (15) programmed Caco-2 cells to constitutively express COX-2, which increased invasiveness compared with control cells. This phenotypic change was associated with an increase in the activation of MMP-2, as determined using gelatin zymography, and an increase in MMP-1 mRNA. Both effects were reversed by the nonspecific NSAID metabolite sulindac sulfide (15). Tomozawa et al. (10) injected MC-26 cells into the tail vein of BALB/c mice and demonstrated that the i.p. injection of JTE-522, a selective COX-2 inhibitor, decreased the number of lung metastases. The authors concluded that selective COX-2 inhibition might be effective in decreasing the hematogenous metastasis of CRC (10).

Using a cell invasion/migration assay that employs Matrigel-coated

Fig. 2. Effect of rofecoxib treatment on mouse splenic tumor COX-1 and -2, cyclin D1, β-catenin, MMP-2 and -9, and VEGF expression. Splenic tumors from rofecoxib-treated and control mice were homogenized, separated by SDS-PAGE, transferred to nitrocellulose, and blotted with COX-1, COX-2, cyclin D1, β-catenin, MMP-2 and -9, and VEGF antisera, with detection by enhanced chemiluminescence. The amounts of COX-1, COX-2, β-catenin, and VEGF were determined by image quantification using a charge-coupled device camera as indicated in “Materials and Methods.” The area of absorbance for known quantities of COX-1, COX-2, β-catenin, and VEGF proteins were used to assess approximate amounts of these proteins in splenic tumor samples. Values represent the mean ± SE for nine animals in each treatment group for COX-1 (A), COX-2 (B), cytoplasmic β-catenin (D), and cytoplasmic VEGF (G), and for five animals in each group for Cyclin D1 (C) and MMP (E and F) proteins. To the left of each bar graph pair, a representative immunoblot analysis from five animals in each treatment group for each protein examined, as well as a representative immunoblot of β-actin. O.D., absorbance.
chambers, we have recently reported that the COX-2 inhibitor NS-398 decreased MC-26 cell migration by \( \sim 60\% \), indicating that COX-2 inhibition may decrease the metastatic potential of CRC, at least in part, by inhibiting cellular invasion (29).

As stated previously, CRC usually presents at an advanced stage and is the second leading cause of cancer deaths in the United States. Up to 50% of patients will die of their disease within 5 years from diagnosis (4). Whereas the 5-year survival of early-stage CRC (Dukes A or B) is relatively high, only \( \sim 40\% \) of those individuals with advanced stages (Dukes C and D) will be alive 5 years after the initial diagnosis (4, 6). In a recent study by Saltz et al. (5), of 683 patients with refractory CRC, a combination of CPT-11 and 5-FU/LV improved survival by only 2.2 months over either agent alone, from a median of 12.6 months to 14.8 months. Although the addition of CPT-11 to 5-FU/LV did not compromise the quality of life of participants in this study, subsequent studies (30, 31) have reported significant toxicity associated with the use of this intense parenteral drug regimen. In contrast, as p.o. administered drugs, COX-2 inhibitors are convenient and relatively well-tolerated by most individuals and have been reported to incite fewer upper gastrointestinal-related events, such as ulcer-associated hemorrhage (32, 33).

In the present study, we examined the effect of rofecoxib treatment on the growth and metastatic potential of colorectal carcinoma in a mouse model of colorectal metastases. The rofecoxib plasma concentrations in drug-treated mice were equivalent to the trough levels in a human 25-mg anti-inflammatory dose. Remarkably, this relatively low dose of rofecoxib not only decreased the size of the primary CRC tumor but also highly significantly decreased the rate and extent of liver metastasis. A striking difference in mortality was also evident in rofecoxib-treated mice when compared with control mice. We also sought to determine molecular mechanisms that might account for the beneficial effects of rofecoxib observed in this \textit{in vivo} study by examining factors shown in various \textit{in vitro} models to be affected by COX-2 overexpression or inhibition, such as cell proliferation, metastasis, angiogenesis, and immune modulation. Immunoblot analysis of the primary tumors demonstrated decreases in the protein expression of COX-2, \( \beta \)-catenin, cyclin D1, VEGF, MMP-2, MMP-9, and IL-10. Interestingly, a significant increase in IL-12 protein was detected in the primary tumors of mice treated with rofecoxib. Recent studies (34–36) have suggested that these two important cytokines play an important role in modulating tumor activity and that COX-2

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**Fig. 3.** Effect of rofecoxib treatment on mouse splenic tumor PGE\(_2\) (A), IL-10 (B), and IL-12 (C) expression. Splenic tumors from rofecoxib-treated and control animals were homogenized, as described in “Materials and Methods.” The concentrations of splenic tumor PGE\(_2\), IL-10, and IL-12 were determined by quantitative ELISA. Values represent mean \( \pm \) SE for nine animals in each treatment group for PGE\(_2\) and IL-12 and for four animals in each treatment group for IL-10.

**Fig. 4.** Primary tumor and hepatic metastasis at day 14 in a representative control mouse (left) and in one mouse treated with rofecoxib (right).
may alter their balance in vivo. IL-10 has been found to be tumor promoting, whereas IL-12 appears to function as a tumor-suppressing cytokine (36). Huang et al. (34) found that PGE2 production by non-small cell lung cancer was dependent on COX-2 expression and that an increase in PGE2 was associated with the induction of IL-10 and the suppression of IL-12. COX-2 inhibition reversed the balance of these cytokines in the lung cancer microenvironment (34). More recent studies using a murine Lewis lung carcinoma model have corroborated these observations (36).

The prolonged survival of rofecoxib-treated animals in our mouse model of CRC metastasis is most likely the result of multiple mechanisms, including antiproliferative and antiangiogenic effects and enhanced immune surveillance. Reductions in primary tumor size and metastatic potential are associated with decreases not only in COX-2 expression and PGE2 synthesis but also in the lowering of various angiogenic factors and a restoration of the balance between cytokines that favors tumor suppression. These studies in mice provide important information that support the benefit of COX-2 inhibition not only in the prevention on CRC but also in the treatment of this common malignancy. Clinical trials will be necessary to assess the utility of COX-2 inhibitors as adjuvant therapy for early-stage disease and as potential agents, either alone or in combination with more established drugs, for the treatment of refractory CRC.

ACKNOWLEDGMENTS

We are grateful to Merck and Co., USA, for a Vioxx Medical School Grant and to Dr. Ian Rodger for his thoughtful suggestions during the course of these studies and in the preparation of the manuscript.

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COX-2 INHIBITION BY ROFECOXIB IN THE TREATMENT OF CRC


