Colon Carcinoma Cell Growth Is Associated with Prostaglandin E2/EP4 Receptor-evoked ERK Activation*

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Cyclooxygenase (COX) and its prostanoid metabolites have been implicated in the control of cell survival; however, their role as mitogens remains undefined. To better understand the role of prostanoids on cell growth, we used mouse colon adenocarcinoma (CT26) cells to investigate the role of prostaglandin E2 (PGE2) in cell proliferation. CT26 cells express both COX1 and COX2 and metabolize arachidonic acid to PGE2. Treatment with indomethacin, or COX2 inhibitors, prevents PGE2 biosynthesis and CT26 cell proliferation. The anti-proliferative effects of COX inhibition are rescued specifically by treatment with PGE2 or the EP4 receptor-selective agonist PGE2-0-H via phosphatidylinositol 3-kinase/extracellular signal-regulated kinase (ERK) activation, thus providing a functional link between dylinositol 3-kinase/extracellular signal-regulated kinase (ERK) activation, and EP4-mediated ERK signaling. Indomethacin or COX2 inhibitors, but not COX1 inhibitors, reduced the size and number of CT26-derived tumors in vivo. These inhibitory effects are paralleled by marked declines in the levels of tumor PGE2, suggesting that their anti-tumor effects are directly associated with the inhibition of COX2 enzymatic activity. The described anti-tumor effects of indomethacin are evident whether it is administered at the time of, or 7 days after, tumor cell injection, suggesting that it has tumor preventive and therapeutic actions. Furthermore, the observation that indomethacin increases the survival rates of tumor-bearing mice, even after withdrawal of the drug, indicates that its anti-tumor effects are long lasting and that it may be potentially useful for the prevention and the clinical management of human cancers.

The identification of cyclooxygenase (COX)† as the target for non-steroidal anti-inflammatory drugs (NSAIDs) led to new understandings of its pathophysiological role and of the mechanisms of action of these drugs (1, 2). The discovery of COX2, an isofrom that, although catalytically identical to COX1, shows inducible tissue-selective expression, suggested physiological and pathophysiological roles for the constitutively expressed (COX1) and inducible (COX2) isofroms, respectively (1–4). Studies showing a correlation between NSAIDs and decreased colon cancer incidence, and the demonstration of up-regulated COX2 expression in colon carcinoma, suggested a role for COX2 in the pathophysiology of colon cancer and created new paradigms for the study of the role of prostanoids in cancer (1–3). Nevertheless, despite extensive supporting evidence, direct links between the anti-tumor effects of NSAIDs and COX inhibition are yet to be established (4–9). The potential for non-COX-dependent anti-tumor effects of NSAIDs (6–9), as well as limited evidence available identifying a specific prostanoid(s) as the mediator responsible for the anti-tumor effects of the COX inhibitors (5), raised questions regarding the mechanism of action of NSAIDs and of the role of arachidonic acid, prostanoids, and COXs in human cancer (4–9).

During studies of arachidonic acid metabolism in mouse colon carcinoma (CT26) cells, we observed the rapid biosynthesis of prostaglandin E2 (PGE2). This, and the powerful tumorigenic activity of CT26 cells, prompted us to characterize the role of PGE2 in tumor growth and development. We show here a direct correlation between COX inhibition, reduced PGE2 biosynthesis, and marked reductions in CT26 cell growth in vitro, as well as tumorigenicity in vivo. These data, together with the finding that the anti-proliferative effects of NSAIDs treatment can be selectively reverted by PGE2 via the Prostaglandin Receptor EP4-mediated phosphatidylinositol 3-kinase (PI3K)/extracellular signal-regulated kinase (ERK) activation, provide a direct functional link between PGE2-induced cell growth and EP4-mediated ERK signaling.

MATERIALS AND METHODS

Arachidonic Acid (AA) Metabolism—Serum-free CT26 cells (a gift of Dr. R. Xiang, Scripps Research Institute, San Diego, CA) or young adult mouse colon cells (YAMC) (a gift of Dr. B. Whitehead, Vanderbilt University, Nashville, TN) were incubated with [1,14C]AA (30 μM, final concentration) at 37 °C. After 20 min, the metabolites present in cells and media were extracted, resolved, and quantified by reversed-phase high performance liquid chromatography (10). For inhibition studies, cells were incubated with varying concentrations of indomethacin, NS398 (both from BioMol, Plymouth Meeting, PA) or SC560 (a gift from Monsanto/Searle, Chesterfield, MO) for 2 h prior to AA addition. The eluted products with the retention time of PGE2 were collected and characterized by gas chromatography/mass spectrometry (GC/MS) (10, 11).

Proliferation Assays—CT26 cells (5 × 10^5/96-well plates) were plated in Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum with or without 10 μM indomethacin, or 5 μM SC560, or 5 μM NS398. To
determine the mitogenic activity of PGE2, cells were incubated with or without COX inhibitors in the presence of PGE2 (0–5 μM). To determine the major prostanoid and the receptor involved in the control of CT26 cell proliferation, cells were cultured with or without 10 μM indomethacin, 5 μM SC560, or 5 μM NS398 for 2 h prior to the addition of [1-14C]AA. After 20 min at 37 °C, the reaction products were extracted, resolved, and quantified as described in A.

The mitogen-activated protein kinase/extracellular signal-regulated kinase kinase 1 (MEK1) inhibitor PD98059 (5 μM), the P38 mitogen-activated protein kinase (MAPK) inhibitor (1 μM), and the PI3K inhibitor wortmannin (100 nM) (all from Calbiochem). Two days after, the medium was replaced with fresh media containing [3H]thymidine (10 μCi/ml), and the cells were incubated for another 48 h. Cells were then processed as described (12). Three to four independent experiments with quadruplicate samples were performed. Manual cell counts paralleled the results of [3H]thymidine incorporation (not shown).
Primary Tumor Growth—Experiments were performed according to institutional animal care guidelines. Male BALB/c mice (5 weeks old; 18–20 g of body weight) were given four dorsal subcutaneous injections of CT26 cells (5 × 10^5/site injection) as described (13). In some experiments, mice injected with CT26 cells were treated with indomethacin (2.5 mg/kg body weight, intraperitoneal injection daily), NS398 (2.5 mg/kg/body weight, intraperitoneal injection every other day), or SC560 (30 µg/injection water) starting at the time of, or 1 week after, cell injection; tumors were harvested after 14 days of growth (see Fig. 4A). For recovery experiments (see Fig. 5A), mice received indomethacin at the time of, or 1 week after, cell injection. Two weeks after cell injection, indomethacin treatment was stopped, and the animals were kept for an additional 10 days. Tumors (24 days total growth) were then harvested, and their volumes were measured and expressed as mm^3 of total tumor volume/mouse.

Measurement of PGE2, and cAMP Levels—Tumors or cells were homogenized and lipids were extracted with methanol containing 4 ng of [3H]PGE2 (Cayman Chemicals). PGE2 levels were then quantified as described (14, 15).

CT26 cells were incubated for 16 h in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 10 µm indomethacin, followed by serum-free medium containing 10 µm indomethacin and 0.5 mCi [3H]thymidine for 30 min. Cells were then incubated for 10 min with PGE2, PGE1, butaprost, or forskolin (1 µm each), washed with PBS, and after adding HCl (0.1 M final concentration), the cells were scraped and lysed; cAMP levels (ng/mg total protein) were determined by enzyme-linked immunosorbent assay (CAMP kit, Cayman Chemicals), according to the manufacturer’s instructions (16).

RT-PCR Analysis—Total RNA was purified from CT26 cells or mouse kidneys using TRIZol reagent (Invitrogen). RNA samples were reverse-transcribed using a Superscript II RT kit and oligo(dT) (12–18 bp). cDNAs were amplified using the following EP-type subtype selective primers: EP1 (710 bp) sense, 5’-ccagcagctccaaatcactg-3’, antisense, 5’-ggaggtgtcctgctgagat-3’, EP2 (507 bp) sense, 5’-cgcggggtctcggggaa-3’, antisense, 5’-gttcgactgcaaggtgtagt3’, EP3a (517 bp) sense, 5’-ctgttgctgatgctgccg-3’, antisense, 5’-tgtgttgctgatgctgccg-3’, EP3b (724 bp) sense, 5’-atacctgctgctgctgccg-3’, antisense, 5’-tgttgttgctgctgctgccg-3’.

Immunohistochemistry—Immunohistochemistry on frozen tumor sections (7 µm each) was done using rat anti-mouse CD31 (1:100, PharMingen) or rabbit anti-mouse PCNA (1:100, Santa Cruz Biotechnology) followed by horseradish peroxidase-conjugated goat secondary antibody to rat or rabbit IgG (1:200, Jackson ImmunoResearch) and Sigma Fast diaminobenzidine chromogenic tablets (Sigma). CD31-positive structures were then imaged and processed as described (17).

Western Blot Analysis—To evaluate ERK and Akt phosphorylation, CT26 cells were cultured for 24 h in serum-free medium before treatment with PGE2, PGE1, indomethacin (1 µM each), or 2% fetal calf serum (to mimic the conditions used for proliferation; see above) with 10 µm indomethacin and the different inhibitors mentioned above in the Materials and Methods. Western Blot Analysis—To evaluate ERK and Akt phosphorylation, CT26 cells were cultured for 24 h in serum-free medium before treatment with PGE2, PGE1, indomethacin (1 µM each), or 2% fetal calf serum (to mimic the conditions used for proliferation; see above) with 10 µm indomethacin and the different inhibitors mentioned above in the Materials and Methods.

RESULTS
CT26 Cells Biosynthesize PGE2—To explore the enzymology of AA metabolism by CT26 cells, we incubated the cells with radiolabeled AA and compared their AA metabolite profiles with those generated by cultured YAMC cells. As shown in Fig. 1A, although YAMC do not metabolize exogenous AA, CT26 cells actively oxidized the fatty acid to a product (98% of total AA metabolism) with the high performance liquid chromatography retention time of authentic PGE2 (16.5 min). Subsequent chromatographic and GC/MS analyses confirmed that CT26 cells... 

Fig. 2. COX inhibitors block CT26 cell proliferation, which can be rescued by PGE2. A–C, CT26 cells (5 × 10^5 cells/well) were plated onto 96-well plates in Dulbecco’s modified Eagle’s medium containing 2% fetal calf serum and indomethacin (A), SC560 (B), or NS398 (C) at the indicated concentrations. For recovery experiments (see Fig. 5A), mice received indomethacin at the time of, or 1 week after, cell injection. Two weeks after cell injection, indomethacin treatment was stopped, and the animals were kept for an additional 10 days. Tumors (24 days total growth) were then harvested, and their volumes were measured and expressed as mm^3 of total tumor volume/mouse.
metabolized AA to PGE$_2$ (19 ± 5 nmol/min/mg of cell protein) as the only detectable product (Fig. 1A).

Western blot analysis demonstrated the presence of both COX1 and COX2 in lysates isolated from CT26 cells, whereas only COX1 was clearly evident in YAMC cells (Fig. 1B). It is interesting that similar levels of COX1 expression in these two cell types, YAMC are unable to support prostanoid biosynthesis (Fig. 1B). The fact that most of the AA is recovered (Fig. 1A) indicates that non-tumorigenic YAMC cells, COX activity is under the control of additional, yet to be characterized regulatory mechanisms. To dissect the roles of COX1 and COX2 in PGE$_2$ formation by CT26 cells, we incubated them with AA in the presence of either a nonspecific COX inhibitor (indomethacin) or a COX1-specific (SC560) or COX2-specific (NS398) inhibitor. As shown in Fig. 1C, 10 μM indomethacin blocked all cellular AA oxidation and PGE$_2$ biosynthesis almost completely. On the other hand, only partial inhibition of PGE$_2$ biosynthesis was achieved at 5 μM SC560 and 10 μM NS398 (70 and 85% inhibition, respectively), and the extent of inhibition did not increase with higher doses. All three inhibitors blocked AA metabolism and PGE$_2$ formation without changing the metabolite profile of the enzyme (Fig. 1C). These results show that in CT26 cells, COX1 and COX2 are capable of metabolizing added AA to PGE$_2$.

To determine whether CT26 cells metabolize endogenous pools of AA and the role of the COX isoforms in PGE$_2$ formation, we quantified (by GC/MS) the levels of endogenous PGE$_2$ in CT26 cells. In the absence of external stimuli, CT26 cells produce significant amounts of PGE$_2$ (58.5 ± 2.9 ng/mg of total protein, n = 10), most of which is secreted into the media (>90% of the total). Endogenous PGE$_2$ synthesis is inhibited by 10 μM indomethacin (7.5 ± 0.9 ng/mg total protein, n = 8), 5 μM NS398 (14.95 ± 1.5 ng/mg total protein, n = 5), or 5 μM SC560 (16.1 ± 3.3 ng/mg total protein, n = 5).

**PGE$_2$ Induces CT26 Cell Growth**—Because PGE$_2$ has been implicated in cell survival (17) and CT26 cells generate high levels of PGE$_2$ (Fig. 1A), we determined the effect of COX inhibitors on cell proliferation. All three COX inhibitors decreased CT26 cell growth in a dose-dependent manner (Fig. 2, A–C), with maximal inhibition obtained between 5–10 μM SC560 or NS398 and 5–20 μM indomethacin, all doses shown to block PGE$_2$ formation (Fig. 1C). Thus, growth of CT26 cells is equally sensitive to COX1 or COX2 inhibition, and the source of PGE$_2$ is not a determining factor for its mitogenic activity.

To determine whether exogenous PGE$_2$ could rescue the cells from COX inhibition, CT26 cells were grown in the presence of 10 μM indomethacin, 5 μM SC560, or 5 μM NS398 added alone or in combination with PGE$_2$ (ranging from 0 to 5 μM) (Fig. 2, D–F). Exogenous PGE$_2$ rescued the growth of cells treated with the COX inhibitors, demonstrating that PGE$_2$ formation was sufficient to rescue cell proliferation and that COX was the target for the inhibitors. The lack of a PGE$_2$ effect upon the growth of cells incubated in the absence of inhibitors (Fig. 2, D–F) suggested that its endogenous levels are sufficient to support growth under steady-state conditions.

To determine the selectivity of the PGE$_2$ mitogenic activity, cells were incubated in the presence of 10 μM indomethacin together with PGE$_2$, PGD$_2$, PGF$_{2α}$, PGJ$_2$, or cicaprost (a prostacyclin receptor agonist) (1 μM each), and their ability to rescue cell growth from COX inhibition was evaluated. Only PGE$_2$ restored proliferation in indomethacin-treated cells to levels similar to those of untreated controls (Fig. 3A), showing that its effects were selective.
CT26 Cell Growth Is Associated with EP4 Receptor-mediated Akt/ERK Activation—Many of the cellular functions of PGE2 are mediated by a family of G protein-coupled receptors, namely EP1, EP2, EP3, and EP4 (18), of which CT26 cells express the EP1, EP2, and EP4 subtypes (Fig. 3B). To characterize the role of these receptors in PGE2-induced mitogenesis, cells were incubated with 10 μM indomethacin alone or in combination with the kinase inhibitors indicated together with PGE2 or PGE1-OH (1 μM each), and proliferation was measured as described under “Materials and Methods.” Note that neither PGE2 nor PGE1-OH was able to rescue indomethacin-treated cells when the ERK or PI3K pathways were inhibited. D, cell lysates obtained from cells incubated as described in C were analyzed by Western blot analysis to determine the phosphorylation levels of the kinases indicated after addition of the specific kinase inhibitors.

It has been proposed that PGE2 stimulation of the EP4 receptors leads to phosphorylation of ERK through a PI3K-dependent mechanism (19). For this reason, we determined the ability of PGE2 and PGE1-OH to activate these two kinases in CT26 cells. Incubation of cells with PGE2 (1 μM) markedly increased ERK and Akt (a PI3K substrate) phosphorylation (Fig. 4B). Similarly, phosphorylation of these two kinases was also observed after treatment with PGE1-OH, but not butaprost (used as the negative control), indicating that the EP4 receptor mediates the effects of PGE2. In addition, our data parallels the finding that phosphorylation of Akt is observed in CT26 cells after PGE2 stimulation (20).

To confirm that the EP4-mediated phosphorylation of PI3K/ERK was necessary to support CT26 cell proliferation, cells were incubated with 10 μM indomethacin in the presence of PD98059 (a MEK1/ERK inhibitor), wortmannin (a PI3K inhibitor), and a p38 MAPK inhibitor (used as the negative control), and the ability of PGE2 or PGE1-OH (1 μM each) to rescue cell proliferation was evaluated. As shown in Fig. 4C, neither PGE2 nor PGE1-OH was able to rescue the proliferation of indomethacin-treated CT26 cells when the ERK or PI3K pathways were inhibited. In contrast, both prostanooids were able to rescue indomethacin-treated cells cultured in the presence of a p38 kinase inhibitor (Fig. 4C) or PKA or PKC inhibitors (not shown), suggesting that these kinases are not involved in the EP4-mediated cell proliferation. Cell lysates derived from cells incubated as described above were analyzed by Western blot analysis. As shown in Fig. 4D, phosphorylation of ERK, Akt, or p38 was completely inhibited after the addition of the specific inhibitors, and no changes in phosphorylation were observed after the addition of PGE2 or PGE1-OH.

Effects of COX Inhibition on Tumor Growth—It has been shown recently that CT26-derived tumors express both COX1 and COX2 (20), suggesting that they could be sensitive to both COX1- and/or COX2-specific inhibition. For this reason, we determined the effects of COX inhibition on the size and number of tumors derived from the injection of CT26 cells in BALB/c mice (13). Mice were treated at the time of cell injection or 1 week after cell injection with indomethacin (Fig. 5A), and tumor growth was compared with untreated control mice. Mice were sacrificed 14 days after cell injection, and tumor number
and volume were evaluated. Indomethacin caused dramatic decreases in tumor number (Fig. 5B) and volume (Fig. 5C), and its anti-tumorigenic effects appeared to be independent of the time of treatment initiation (Fig. 5, B and C). Indomethacin, administered 1 week after tumor cell injection, arrested tumor growth at levels similar to those observed in control mice sacrificed 1 week after tumor cell injection (not shown). Thus, depending upon the time of treatment initiation, indomethacin can both prevent tumor uptake and development and inhibit tumor growth. To determine whether the effects of indomethacin on tumor growth correlated with COX inhibition and a resultant decrease in PGE$_2$ levels, endogenous PGE$_2$ levels in tumors from untreated or indomethacin-treated mice were evaluated by GC/MS. Indomethacin treatment caused dramatic reductions in tumor PGE$_2$ levels, thus providing a strong demonstration of a direct link between the biological response to the inhibitor, reductions in COX activity, and PGE$_2$ biosynthesis (Fig. 5D).

To identify the COX isoform involved in tumor growth, tumor-bearing mice were treated with SC560 or NS398 using a protocol similar to that in Fig. 5A. Notably, NS398 caused a significant reduction in tumor growth (Fig. 5, B and C) and PGE$_2$ levels (Fig. 5D), independently of the time of treatment. In contrast, SC560 caused a mild reduction in tumor size with no changes in endogenous PGE$_2$ levels only when administered at the time of cell injection (Fig. 5, B–D). Although the effects of NS398 were less pronounced than those of indomethacin, they suggest that COX2 plays the dominant role in tumor PGE$_2$ biosynthesis and that COX2 and PGE$_2$ are mostly responsible for regulating CT26 cell growth in vivo.

We then determined the effect of COX inhibition on tumor vascularization, growth, and apoptosis. Treatment with indomethacin and NS398, but not SC560, resulted in decreased tumor vascularization (Fig. 6, A and B), reduced proliferation (Fig. 6, C and D), and increased apoptosis (Fig. 6, E and F),
Fig. 7. The anti-tumor effects of indomethacin last also upon withdrawal of the drug. A, schematic representation of the in vivo protocol. CT26 (5 × 10⁶ cells/mouse) were injected into BALB/c mice (10 mice/group). Tumor-bearing mice were then divided into three groups: treated for 1 week with indomethacin, starting 1 week after cell injection (a); treated for 2 weeks with indomethacin starting at the time of cell injection (b); and untreated controls (c). Two weeks after cell injection, the mice were switched to an indomethacin-free regime, and their survival rate was compared with those of untreated mice. All animals were sacrificed 24 days after cell injection because the survival rate of mice sacrificed 24 days after CT26 cell injection. Values are averages calculated from 10 mice/treatment. C, PGE₂ levels in tumor extracts. PGE₂ levels were determined as described under “Materials and Methods” and expressed as ng/mg total protein. Values are from 10 tumors/treatment. Differences between mice untreated and treated with indomethacin (*) were significant with p < 0.05.

which parallel the changes observed in tumor volume (Fig. 5, B and C).

The Effects of Indomethacin Are Long Lasting—An important issue for drug anticancer therapy is the duration of the beneficial effects after drug withdrawal. To address this, we developed the survival protocol shown in Fig. 7A. Mice were injected with CT26 cells and kept untreated (Fig. 7A, a) or treated with indomethacin starting at the time of Fig. 7A, b), or 1 week after (Fig. 7A, c), cell injection. Two weeks later, indomethacin treatment was stopped, the mice were kept for an additional 10 days, and then sacrificed. At the time of sacrifice, mice that received indomethacin for 1 or 2 weeks showed 100% survival, compared with the 50% survival rate observed in untreated controls. Regardless of either the length of indomethacin treatment or drug withdrawal, the indomethacin-treated mice showed marked reductions in tumor volume (Fig. 7B) when compared with their untreated counterparts. Additionally, in indomethacin-treated mice, the PGE₂ levels in tumors remained reduced, and these reductions were proportional to the length of treatment with the drug (Fig. 7B, a and C). These results show that the effects of COX inhibition are long lasting, are evident even after the withdrawal of the inhibitor, and result in markedly increased survival.

DISCUSSION

The evidence that up-regulated COX expression results in increased AA metabolism or in-tumor prostanooid levels is limited (4–8, 21); attempts to establish direct links between the anti-tumor effects of NSAIDs and COX inhibition have yielded mixed results (9, 22). A role for COX2 in colon cancer was indicated by studies with the Apc-min and APC<sup>−/−</sup> mouse, two models that spontaneously develop benign intesti-
We also observed dramatic reductions in tumor vascularization after treatment with indomethacin or NS398 but not with SC560. This reduction could be because of the direct effects of these inhibitors upon endothelial cell function, because endothelial cells express both COX1 and COX2 (34), and because specific or nonspecific COX inhibition prevents endothelial cell spreading, migration, and angiogenesis in vivo (35–37). Alternatively, COX inhibitors could reduce new blood vessel formation by down-regulating the synthesis of angiogenic factors by tumor cells. In this context, COX2 overexpression in cultured colon carcinoma cells leads to COX2-dependent production of angiogenic factors (37).

In conclusion, we provide evidence that the effects of indomethacin on PGE2 biosynthesis and tumor growth are therapeutic, preventive, and lasting, as no further tumor growth was evident after cessation of treatment (Fig. 7). These results, in combination with the documented increased survival rate in the indomethacin-treated mice, could have profound clinical implications for cancer treatment and prevention.

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