Modulation of Apoptosis and Bcl-2 Expression by Prostaglandin E$_2$ in Human Colon Cancer Cells

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ABSTRACT

Previously, we have shown that forced expression of prostaglandin endoperoxide synthase-2 (also called cyclooxygenase (COX) 2) leads to inhibition of programmed cell death in intestinal epithelial cells. More recently, we have demonstrated that growth of human colon cancer xenografts is inhibited by treatment with a highly selective COX-2 inhibitor in tumors that express COX-2 (HCA-7) but not in those that lack COX-2 expression (HCT-116). To explore the biochemical mechanisms involved in these effects, we have evaluated the role of COX-2-derived eicosanoids on programmed cell death in human colon cancer cells. Here we report that PGE$_2$ treatment of human colon cancer cells leads to increased clonogenicity of HCA-7, but not HCT-116 cells. Treatment with a highly selective COX-2 inhibitor (SC-58125) decreases colony formation in monolayer culture and this growth inhibition was reversed by treatment with PGE$_2$. Additionally, PGE$_2$ inhibits programmed cell death caused by SC-58125 and induces Bcl-2 expression, but did not affect Bcl-x or Bax expression in human colon cancer (HCA-7) cells. Therefore, decreased cell death caused by PGE$_2$ would enhance the tumorigenic potential of intestinal epithelial cells. Thus, these results may help to explain a component of the mechanism by which COX inhibitors prevent colorectal cancer in humans.

INTRODUCTION

Numerous epidemiological and clinical studies have been published that demonstrate a 40–50% reduction in the relative risk of colorectal cancer in humans who take NSAIDs on a regular basis (1). The precise mechanism for the chemoprotective effects of NSAIDs is unknown. However, one common effect of NSAIDs is the inhibition of prostaglandin synthase enzymes. It seems clear that inhibition of prostaglandin production results in the anti-inflammatory effects of NSAIDs (2). Several reports have noted elevated levels of prostaglandins in colorectal carcinoma tissue when compared to normal colonic mucosa (3–6). A recent study found a statistically significant increase in PGE$_2$ in 21 surgically excised colorectal cancers compared to the accompanying normal colorectal mucosa (7). Therefore, the COX pathway of arachidonic acid metabolism may play an important role in the chemoprotective effect of NSAIDs.

Two isoforms of prostaglandin synthase have been identified and are often referred to as COX-1 and COX-2 (8). COX-2 levels have been reported to increase dramatically in human colorectal adenocarcinomas (9–12), in adenomas taken from APC mutant mice (13, 14), and in intestinal tumors from carcinogen-treated rats (15). Forced expression of the COX-2 gene in intestinal epithelial cells results in resistance of the cells to undergoing apoptosis and increased levels of Bcl-2 protein (16). Additionally, the COX-2 pathway has been used as a target for treatment with highly selective COX-2 inhibitors, resulting in a reduction of size and number of premalignant and malignant lesions in a number of animal models (14, 17, 18). The molecular events that result in the reduction of tumor size and number via inhibition of the COX-2 pathway is an area of intense research interest. Here, we report the results of studies aimed at understanding the molecular mechanism(s) for the reduction in tumor growth via inhibition of COX-2.

We have evaluated a number of human colon cancer cell lines and have found that some maintain high levels of constitutive COX-2 expression, whereas other cell lines lack COX-2 expression completely (18). In nontransformed intestinal epithelial cells, COX-2 is rapidly induced by treatment with growth factors or tumor promoters but is expressed transiently (19, 20). COX-2 was initially cloned as a transiently expressed immediate early gene in phorbol ester-stimulated fibroblasts or in Src-transformed chicken embryo fibroblasts (21, 22). One of the human colon cancer cell lines we have studied (HCA-7) maintains high constitutive COX-2 expression and prostaglandin production in long-term culture (18, 23). Recently, we reported that treatment of HCA-7 cells with a highly selective COX-2 inhibitor blocked growth of HCA-7 xenografts in nude mice (18). Here, we show that treatment of the HCA-7 cells with a highly selective COX-2 inhibitor, SC-58125, increases the relative rate of programmed cell death, and that this is reversed by exogenously administered PGE$_2$. Additionally, PGE$_2$ treatment of HCA-7 cells resulted in a 4–5-fold increase in Bcl-2 protein levels and an activation of MAPK. Therefore, the mechanism by which COX-2 expression leads to inhibition of apoptosis may relate to the modulation of Bcl-2 expression by the COX-2-derived eicosanoid PGE$_2$.

MATERIALS AND METHODS

Cell Culture. Human colon cancer lines (HCA-7 and HCT-116) were maintained in McCoy’s 5A medium supplemented with 10% FCS as described previously (24, 25). The MAPK kinase inhibitor (26), PD 098059, was obtained from Calbiochem (San Diego, CA).

Prostaglandin Production. For these experiments, subconfluent cell cultures were established and treated with different concentrations of SC-58125 [f-(4-methylsulfonyl)phenyl]-3-trifluoromethyl-5-(4-fluoro)phenyl]pyrazole; G. D. Searle and Co., St. Louis, MO] for 24 h. Serum-free medium was supplemented with 15 μM arachidonic acid (Cayman Chemical, Ann Arbor, MI) 1 h prior to collection of samples for eicosanoid measurements. PGE$_2$ was quantified in medium from cell incubations using stable isotope dilution techniques using gas chromatography/negative ion chemical ionization mass spectrometry as described (19, 23). The limit of sensitivity for detection of either PGE$_2$ is 4 pg/ml, and the results are expressed as ng of PGE$_2$ per 10$^6$ cells.

Immunoblotting. Immunoblot analysis of cell protein lysates was performed as described previously (27). Briefly, the cells were lysed for 30 min in radioimmunoprecipitation assay buffer (1 X PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mm sodium orthovanadate); and then clarified cell lysates were probed with an anti-human COX-2 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and developed by the enhanced chemiluminescence system.

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#The abbreviations used are: NSAID, nonsteroidal antiinflammatory drug; COX, cyclooxygenase; PGE$_2$, prostaglandin E$_2$; MAPK, mitogen-activated protein kinase.

362
Quantiﬁcation was carried out by video densitometry. The Bcl-2, Bax, and Bcl-x antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used at a dilution of 1:250, and the antiactive MAPK antibody (Promega, Madison, WI) was used at a dilution of 1:1000.

**Colonie Morphology.** HCA-7 or HCT-116 cells were suspended in 0.5 ml of 1:2 diluted Matrigel (Collaborative Biomedical Products, Bedford, MA). The mixture was then plated into 24-well plates and incubated for 2 weeks. The colonies were ﬁxed with 10% formaldehyde and stained with H&E.

**Plating Efﬁciency Assay.** HCA-7 or HCT-116 cells were plated in T-25 cell culture ﬂasks at a density of 3000 cells ﬂask, with McCoy’s medium containing 10% FCS. SC-58125 or PGE2 was added daily to selected cells, and the medium was replaced every 2 days. Cells were incubated for 14 days, and then colonies were visualized by staining with 0.2% methylene blue and counted manually.

**Tumor Xenografting.** Cells were suspended in 0.2 ml of DMEM and were injected into the dorsal s.c. tissue of athymic nude mice (Sprague-Dawley nu/nu; Harlan Sprague Dawley, Inc., Indianapolis, IN). The animals were sacriﬁced 42 days following implantation of the cells. The tumors were collected and subjected to routine histological examination.

**Apoptosis Measurements.** Subconﬂuent HCA-7 cultures were established in 35-mm plates. PGE2 (0.7 μM) was added to the cells 2 days prior to SC-58125 treatment. The cells were then exposed to SC-58125 (25 μM) for 48 h. The floating cells and attached cells were collected separately and stained with a DNA-speciﬁc ﬂuorochrome bis-benzimide trihydrochloride (Hoechst 33258; Sigma Chemical Co.). After ﬁxing with glutaraldehyde, the cells were stained in 167 μM bis-benzimide and observed under fluorescent microscopy, as described previously (16). Cells with three or more nuclear chromatin fragments were considered positive for apoptosis. The results were expressed as a percentage of total cells staining positive for apoptosis.

**RESULTS**

**COX Expression and PGE2 Production in Colon Cancer Cells.** We evaluated PGE2 production by HCA-7 and HCT-116 colon cancer cells, and as previously reported (18, 23), the HCA-7 cells produce signiﬁcant amounts of PGE2, whereas the HCT-116 cells lack prostaglandin production (Fig. 1A). Addition of a highly selective COX-2 inhibitor (SC-58125) to the cell culture medium resulted in a dose-dependent decrease in PGE2 production (Fig. 1A). We observed high levels of constitutive expression of COX-2 in the HCA-7 cells but no expression of COX-2 protein in HCT-116 cells (Fig. 1B). COX-1 expression was undetectable in both HCA-7 and HCT-116 cells by immuno blotting assays. In colony growth assays, SC-58125 (25 μM) treatment markedly reduced the number of HCA-7 colonies that develop but had no effect on HCT-116 colony formation (Fig. 1C).

Interestingly, the HCA-7 cells form differentiated, cyst-like structures when plated in extracellular matrix components, whereas the HCT-116 cells form solid noncystic colonies (Fig. 1D, 1 and 3). Both of these cell lines will grow into solid tumors when xenografted into nude mice (18). However, the HCA-7 tumors are much more well differentiated than the HCT-116 tumors (Fig. 1D, 2 and 4) and maintain high levels of COX-2 expression, even following xenografting (18).

**Effect of PGE2 Treatment on Clonogenicity of HCA-7 and HCT-116 Cells.** Because the HCA-7 cells produce signiﬁcant amounts of PGE2 (28 ng/ml), we then evaluated the effect of PGE2...
treatment on colony formation in monolayer culture. For this experiment, 3000 cells were seeded in T-25 flasks, and PGE2 was added daily at concentrations of 0.3, 1.0, 3.0, and 10.0 μM in fresh medium. We observed a 1.5-fold increase in HCA-7 colony number following 0.3, 1.0, and 3.0 μM PGE2 treatment for 14 days (Fig. 2B) and a slight decrease in HCT-116 colony number (Fig. 2A). Interestingly, the highest dose of PGE2 (10 μM) did not significantly increase HCA-7 colony number but did decrease HCT-116 colony number. We also determined the dose dependence of this effect and found that the HCA-7 colony number increased at 0.3–3.0 μM PGE2, with a slight decrease in HCT-116 colony number, at all of the concentrations used in this experiment. PGE2 treatment also caused a significant increase in HCA-7 colony size (data not shown). We are currently evaluating the PGE2 receptor status of these two cell lines to determine whether the effect on tumorigenicity in HCA-7 cells is related to PGE2 receptor activation. HCT-116 cells may express a different set of E2 prostaglandin (EP) receptor isoforms than do the HCA-7 cells, and this could help to explain the altered responsiveness of HCT-116 versus HCA-7 cells.

Effect of COX-2 Inhibition on Colony Number Can Be Reversed by PGE2 Treatment. Because we had shown previously that both HCA-7 colony and tumor growth were inhibited by treatment with a highly selective COX-2 inhibitor, we next sought to determine whether this growth inhibition could be overcome by addition of COX-2-derived eicosanoid products to the cells. For this experiment, the plating efficiency assay was used. HCA-7 cells were treated with various concentrations of SC-58125, and colony number was measured after 14 days of treatment. We observed a dose-dependent decrease in colony number by 16–61% at 5–25 μM SC-58125 treatment (Fig. 3). Importantly, growth inhibition could be overcome with PGE2 (0.7 μM) administered to HCA-7 cells treated with SC-58125. These results indicate that PGE2 production via the COX-2 pathway could be an important component in maintaining colony growth.

Fig. 2. Effect of PGE2 treatment on HCA-7 and HCT-116 colony number. A, HCT-116 colony number in response to treatment with PGE2. Cells were plated in T-25 cell culture flasks at a density of 3000 cells/flask, with McCoy's medium containing 10% FCS. Cells were incubated for 14 days, and then colonies were visualized by staining with 0.2% methylene blue and counted manually. The results are expressed as means (bars, SE), and each experiment was repeated three times. B, HCA-7 colony number in response to treatment with PGE2. Conditions were identical to those described above for the HCT-116 cells.

Effect of COX-2 Inhibition and PGE2 Treatment on Programmed Cell Death. The size and number of tumor colonies can be affected by either increased proliferation of cells or decreased rates of programmed cell death. In other cell culture systems, increased COX-2 expression or PGE2 treatment leads to inhibition of programmed cell death (16, 28, 29). However, it is well established that PGE2 induces B cells to undergo apoptosis (30), so the net effect of PGE2 treatment depends on the particular cell type being evaluated. Therefore, we decided to determine the effect of PGE2 treatment on programmed cell death in HCA-7 cells that are of epithelial origin. To evaluate the role of apoptosis in the maintenance of colony number, we determined the percentage of HCA-7 cells undergoing apoptosis in the presence and absence of a COX-2 inhibitor, SC-58125. We found, by nuclear staining and evaluation of DNA fragmentation patterns, that the cells that became detached from the cell culture dish were undergoing programmed cell death (Fig. 4A). We then determined the percentage of cells undergoing apoptosis in the presence or absence of a SC-58125 and found a 1.5–1.6-fold increase in apoptosis following SC-58125 treatment (Fig. 4B), which was reversed by treatment with 0.7 μM of PGE2. Although the change in percentage of cells undergoing apoptosis is relatively small, over a prolonged period of time, this would amount to a significant reduction in tumor size based on cell kinetic studies (31).

PGE2 Treatment Increases Bcl-2 Levels. To evaluate the molecular mechanisms involved in the PGE2-mediated increase in colony number, we measured the protein levels of Bcl-2, Bcl-x, and Bax. We observed a dramatic (4–5-fold) increase in Bcl-2 protein levels at 8 h following PGE2 treatment, without a significant effect on Bax or Bcl-x (Fig. 5A). Interestingly, we also observed a significant increase in active MAPK following PGE2 treatment, which was reduced markedly by addition of a MAPK kinase inhibitor, PD-098509 (Fig. 5B). In Fig. 5B, we show the results of a Western blot assay using the antibody that only recognizes the active form of the enzyme. At 3–6 h following PGE2 treatment, there is a 4–5-fold increase in the level of active MAPK protein. The temporal pattern for this increase precedes the induction of Bcl-2 expression.

DISCUSSION

Colonic neoplasms are believed to develop through a series of sequential steps over 15–20 years that reflect the progressive accumulation of mutations (32). Early in the transition from normal colonic epithelium to adenomas, mutations of key genes occur, which have been implicated in the transformation process. One such gene is the adenomatous polyposis coli (APC) gene. Inactivating mutations of
this gene are known to cause colorectal cancer in patients with familial adenomatous polyposis syndromes, and truncation mutations in the APC gene occur somatically in a large percentage of colorectal cancers that form spontaneously (33). Recently, it has been demonstrated that disruption of the APC gene in mice leads to increased expression of COX-2 in intestinal tumors (13, 14). Therefore, the increased levels of COX-2 in intestinal tumors could represent an event downstream of an early mutation in a key regulatory gene, such as APC. We and others have shown previously that COX-2 levels are increased in human colorectal adenocarcinomas (9–12). Forced expression of COX-2 in intestinal epithelial cells leads to inhibition of apoptosis and induction of Bcl-2 expression (16). A key question we have addressed here is whether a COX-2-derived eicosanoid product, such as PGE2, could modulate apoptosis and/or Bcl-2 expression.

Because malignant progression in cancer is thought to be related to alterations in the biology of epithelial cells related to genetic defects, it is of interest to determine the effect of COX-2-derived eicosanoids, such as PGE2, on the biology of human colon cancer cells. One obvious cellular process that could be affected involves relative rates of cells undergoing apoptosis. To evaluate this, we determined the effect of PGE2 treatment on the number of colonies that form by plating colonic tumor cells in monolayer culture. In addition to observing an increase in the number of HCA-7 cells in response to PGE2 treatment, there also was a reduction in the percentage of cells undergoing apoptosis. To evaluate the mechanisms involved, we determined that PGE2 treatment led to a significant induction of Bcl-2 expression, but not Bax or Bcl-x. These biochemical changes could explain a reduction in programmed cell death, thus increasing colony number. In addition, PGE2 treatment led to a marked activation of MAPK, which preceded induction of Bcl-2.

Our finding that apoptosis is regulated by treatment with a highly selective COX-2 inhibitor, and that this is reversible upon treatment with PGE2, indicates that COX-derived eicosanoids are likely to play an important role in colorectal carcinogenesis. PGE2 treatment could affect colony size and number by increasing the proliferation rate and/or decreasing the percentage of cells undergoing programmed cell death by increasing Bcl-2 levels. Our finding that PGE2 treatment activates MAPK indicates a putative signal transduction pathway by which Bcl-2 expression and other genes could be induced.

Overall, our findings provide additional evidence that the COX-2 pathway may provide an attractive target for developing strategies to prevent colorectal lesions early in the adenoma-to-carcinoma sequence. With the recent development of highly selective COX-2 inhibitors, an evaluation of the effectiveness of these agents in chemoprevention of colorectal cancer should be considered.

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