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Alterations in Cellular Adhesion and Apoptosis in Epithelial Cells Overexpressing Prostaglandin Endoperoxide Synthase 2

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Summary

Prostaglandin endoperoxide synthase 2, also referred to as cyclooxygenase 2 (COX-2), is a key enzyme in the conversion of arachidonic acid to prostaglandins and other eicosanoids. Rat intestinal epithelial (RIE) cells were permanently transfected with a COX-2 expression vector oriented in the sense (RIE-S) or antisense (RIE-AS) direction. The RIE-S cells expressed elevated COX-2 protein levels and demonstrated increased adhesion to extracellular matrix (ECM) proteins. E-cadherin was undetectable in RIE-S cells, but was elevated in parental RIE (RIE-P) and RIE-AS cells. RIE-S cells were resistant to butyrate-induced apoptosis, had elevated BCL2 protein expression, and reduced transforming growth factor β2 receptor levels. The phenotypic changes involving both increased adhesion to ECM and inhibition of apoptosis were reversed by sulindac sulfide (a COX inhibitor). These studies demonstrate that overexpression of COX-2 leads to phenotypic changes in intestinal epithelial cells that could enhance their tumorigenic potential.

Introduction

Arachidonic acid metabolites, collectively referred to as eicosanoids, such as hydroxyeicosatetraenoic acids (HETEs), prostaglandins, thromboxanes, and leukotrienes affect a number of signal transduction pathways that modulate cellular adhesion, growth, and differentiation (Bronstein and Bull, 1993; Eberhart and DuBois, 1995; Marnett, 1992). Prostaglandin endoperoxide synthase (cyclooxygenase [COX]) is a key enzyme in the production of prostaglandins and other eicosanoids. Recently, a second isoform of this enzyme (prostaglandin endoperoxide synthase 2 or COX-2) has been identified, which is inducible by a variety of cytokines, hormones, and tumor promoters (DuBois et al., 1994a; Fletcher et al., 1992; Fu et al., 1990; Geng et al., 1995; Harris et al., 1994; Hla and Neilson, 1992; Jackson et al., 1993; Simmons et al., 1989). The enzymatic activity of both isoforms is inhibited by aspirin and other nonsteroidal antiinflammatory drugs (NSAIDs) (Marnett, 1992; Meade et al., 1993). The gastric side effects associated with intake of these drugs in humans is thought to be related to inhibition of COX-1 (Eberhart and DuBois, 1995), but the clinical effects related to COX-2 inhibition have not been fully evaluated (Klein et al., 1994; Seibert et al., 1994). Humans who take aspirin or other NSAIDs on a regular basis have a 40%–50% lower relative risk of colorectal cancer when compared with persons not taking these medications (Giovannucci et al., 1994; Greenberg et al., 1993; Thun et al., 1991, 1993).

The mechanism for reduction of colorectal cancer by NSAIDs is not clear, but NSAIDs are potent inhibitors of tumor formation in rodent models of chemically induced colon cancer (Reddy et al., 1987, 1990, 1992, 1993), and treatment of familial adenomatous polyposis (FAP) patients with NSAIDs results in a reduction in the number and size of adenomas present in the large intestine (Giardiello et al., 1993; Waddell et al., 1989; Waddell and Loughry, 1983; Winde et al., 1993). Also, one NSAID, sulindac, was recently reported to increase the rate of programmed cell death in the colonic epithelium of FAP patients (Pasricha et al., 1995). In a separate study, NSAIDs were also shown to induce apoptosis in chicken embryo fibroblasts (Lu et al., 1995). The precise mechanism for the antitumor activity of these drugs or their effect on apoptosis is presently not understood.

Previous work from our laboratory has demonstrated that the *COX-2* gene is induced following growth factor or tumor promoter stimulation of parental rat intestinal epithelial (RIE-P) cells (DuBois et al., 1994a, 1994b). We have also reported that *COX-2* mRNA levels are markedly increased in 86% of human colorectal adenocarcinomas (Eberhart et al., 1994). However, COX-1 expression is unaffected during colorectal carcinogenesis. Studies from other laboratories have confirmed our findings and shown that COX-2 protein levels are increased in a significant number of colorectal carcinomas as well (Kargman et al., 1995).

The aim of the present study was to evaluate RIE cells for phenotypic and biochemical changes associated with COX-2 overexpression. Gene disruption approaches can be employed to determine the biological effect of a lack of these enzymes. We chose to overexpress COX-2 in intestinal epithelial cells, since COX-1 was not regulated during colorectal carcinogenesis. Cells permanently transfected with a COX-2 expression vector exhibited distinct phenotypic changes as demonstrated by alterations in adhesion to extracullular matrix (ECM) proteins and inhibition of apoptosis following butyrate treatment. We also found that BCL2 levels were high in RIE-S cells compared with RIE-P or RIE-AS controls, which may relate to their resistance to undergo apoptosis. Other biochemical changes in the RIE-S cells included decreased expression of both E-cadherin and transforming growth factor β2 (TGFβ2) receptor. E-cadherin is involved in cell–cell adhesion, and TGFβ2 receptors transduce signals important in modulating programmed cell death.

Our findings suggest that COX-2 overexpression is linked to changes in cellular adhesion and inhibition of apoptosis. Since COX-2 is expressed at high levels in colorectal carcinomas and NSAIDs (like aspirin) decrease the relative rate of colorectal cancer in humans, our results

may offer some insight into the chemoprotective effects of NSAIDs. An improved understanding of the molecular mechanisms underlying these effects may reveal additional molecular targets for new drugs and lead to novel strategies for the development of other cancer chemopreventive agents.

Results

Preparation of RIE Cells That Constitutively Overexpress COX-2

COX-2 is undetectable in quiescent RIE cells, but is transiently induced 6- to 10-fold within 6 hr of growth factor treatment (DuBois et al., 1994a). To examine the effect of COX-2 expression on intestinal epithelial cells, RIE cells were transfected with the eukaryotic expression vector, pCB7, containing the COX-2 coding region in the sense (RIE-S) and antisense (RIE-AS) orientations. We isolated 225 stable transfectants in each group and used 5 clones with high (RIE-S) and low (RIE-AS) prostaglandin production relative to the parental line (RIE-P) for further studies. To evaluate COX-2 expression levels in RIE-S and RIE-AS cells, Western blot analysis and COX assays were carried out. As shown in Figure 1A, the anti-COX-2 antibody detected a protein of the expected molecular mass (69,000 Da) in the sense vector–transfected cells that was absent in the antisense cell lines. In addition, there was a marked increase in arachidonate conversion to eicosanoid products in RIE-S compared with RIE-AS cells, which was inhibited by sulindac sulfide in a dose-dependent manner (Figure 1B). The levels of 6-keto-$PGF_{1\alpha}$ production (an index of PGI2 synthesis), determined as previously described (DuBois et al., 1994b), were 9.58 ± 0.14 ng × 10^{-5} cells for RIE-S without sulindac sulfide, 0.091 ± 0.03 ng × 10^{-5} cells for RIE-S in the presence of 10 μM sulindac sulfide, and 0.043 ± 0.01 ng × 10^{-5} cells for RIE-AS. Previous work has demonstrated that RIE-P cells produce 0.8 ng × 10^{-5} cells of 6-keto-$PGF_{1\alpha}$ (DuBois et al., 1994a). These results demonstrate a greater than 10-fold increase in COX protein levels and enzymatic activity in RIE-S cells compared with RIE-AS cells and that 10 μM sulindac sulfide dramatically inhibited prostaglandin production in these cells.

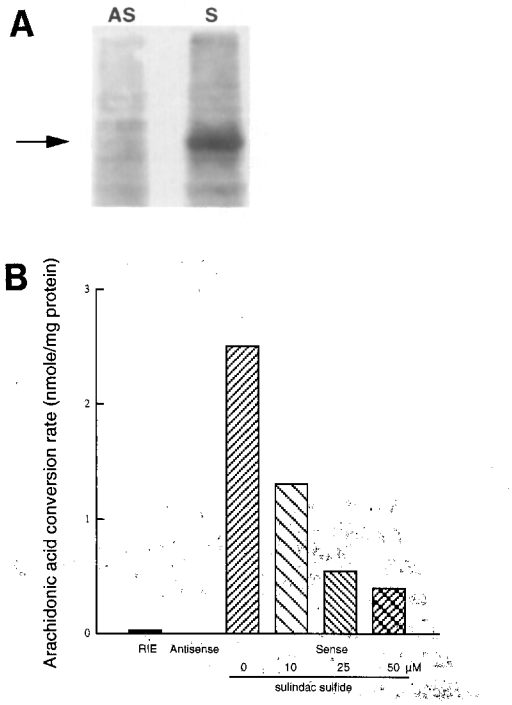

Figure 1. COX-2 Expression and Enzymatic Activity in RIE Cells

(A) Western blot analysis for COX-2 expression. The arrow points to the 69,000 Da COX-2 band. RIE-P cells transfected with sense (RIE-S) and antisense (RIE-AS) oriented *COX-2* cDNA were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were harvested and lysed in RIPA buffer. Protein (100 μg) of the cell lysate samples was separated on 12.5% SDS–polyacrylamide gels. Cellular expression of COX-2 was detected by using a rabbit polyclonal antiserum (DuBois et al., 1994a) by Western blotting procedures following the instructions of the manufacturer (ECL system; Amersham).

(B) COX enzyme assay. The cells were harvested and resuspended in PBS containing 25 μM [^{14}C]arachidonate for 15 min at 25°C with constant mixing. The organic soluble reaction products were extracted into ethyl acetate and separated by reverse-phase HPLC using a μBondapak C_{18} column utilizing an acetonitrile–water gradient. The radioactive metabolites in the eluent were monitored by an on-line scintillation counter. The COX activity was estimated by arachidonic acid conversion rate (nmole/mg protein).

COX-2 Overexpression Causes Increased Binding of RIE Cells to Selected ECM Proteins

Since COX-2 is induced in intestinal epithelial cells by growth factors and tumor promoters, we initially hypothesized that the stable expression of COX-2 might have an effect on mitogenesis, but have demonstrated that mitogenesis is not increased in COX-2-overexpressing cells (data not shown). Therefore, other important aspects of cell growth control and differentiation were evaluated. In intestinal mucosa in vivo, continuously proliferating stem cells are located in the crypt region. Both cellular differentiation and proliferative arrest are induced as intestinal stem cells ascend upward on basement membrane along the crypt–villus axis toward the gut lumen. When the cells reach the surface of the villi, they become fully differentiated, apoptosis is activated, and the cells are shed into the lumen. In rodent small intestine, the entire lifetime of an epithelial cell is only 3–4 days, indicating that the turnover of epithelial cells is rapid. Therefore, cellular differentiation and apoptosis are important factors in maintaining the integrity and function of intestinal mucosa. Alterations is these processes could have significant biological consequences and effect the tumorigenic potential of epithelial cells.

RIE-P cells are derived from normal undifferentiated intestinal crypt cells and do not spontaneously undergo differentiation in culture (DuBois et al., 1994b). Other nontransformed intestinal cells, such as IEC-6 cells, have been reported to show cellular differentiation and proliferative arrest when plated on ECM preparations such as Matrigel, a model basement membrane (Carroll et al., 1988). These results suggested that interactions between epithe-

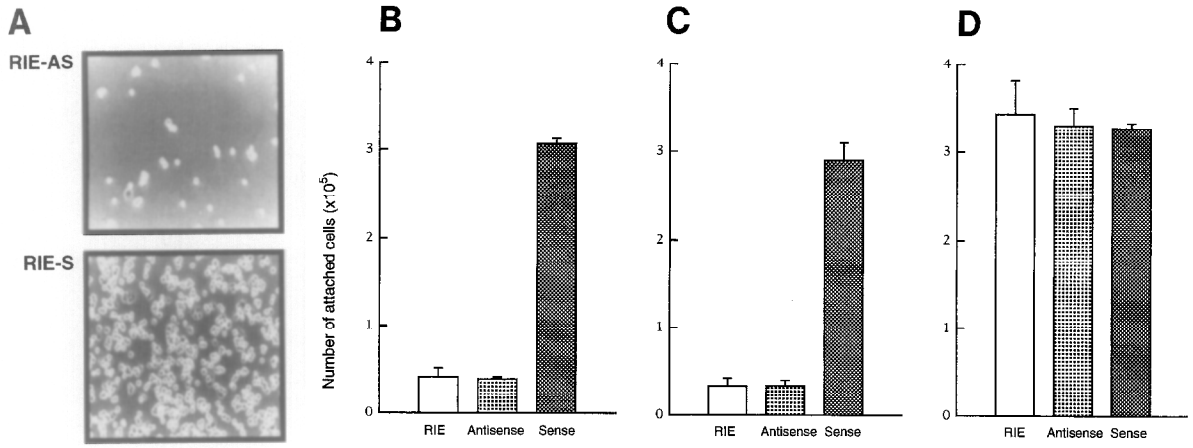

Figure 2. Adhesion of RIE Cells To ECM-Coated Plates

(A) Photomicrograph of RIE-AS and RIE-S cells attached to laminin-coated plates. For this experiment, 4 × 10^5 cells were plated on plates precoated with purified laminin. The plates were incubated at 37°C for 45 min, and the wells were washed to remove unattached cells. Photomicrographs were then taken of the plates using phase-contrast microscopy. Total magnification is 30×.

(B–D) Effect of COX-2 overexpression of cellular attachment to laminin (B), Matrigel (C), and fibronectin (D): 4 × 10^5 cells of each group cultured in media without serum for 3 days were plated into 2 cm^2 wells coated with Matrigel, laminin, or fibronectin. After the plates were incubated at 37°C for 45 min, the wells were washed to remove unattached cells. Attached cells were incubated for 1 hr in dipase and resuspended, and cell counts were determined with a hemocytometer. Five independent clones from each group were tested for phenotypic differences, and they all demonstrated the identical phenotype (data not shown). The results shown here are from studies of one independent clone and were done in quadruplicate.

lial cells and ECM have an important effect on induction of cellular differentiation and apoptosis. Therefore, we explored the ability of RIE cell lines to adhere to selected components of ECM. As shown in Figure 2A, there is a clear phenotypic difference in the RIE-S and RIE-AS cells in their ability to bind to laminin-coated plates.

To quantitate this effect in each group (RIE-P, RIE-AS, and RIE-S), 4 × 10^5 cells were plated on Matrigel-, laminin-, or fibronectin-coated wells, and the number of cells bound to ECM-coated plates over 45 min was determined. As shown in Figures 2B–2D, the number of RIE-S cells that attached to Matrigel and laminin was 6-fold higher than the number of RIE-P and RIE-AS cells. These results suggest that COX-2 expression increased the ability of cells to attach to laminin and Matrigel, but it is of interest that each group of cells bound to fibronectin-coated plates equally well (Figure 2D), indicating that not all components of the extracellular adhesion pathway were affected.

Next, we investigated the kinetics of binding to Matrigel. Three groups of cells (RIE-P, RIE-AS, and RIE-S) were cultured on Matrigel for 14 days, and at various intervals, the number of attached cells were counted. As shown in Figure 3, RIE-S cells continue to grow slowly over the 2 week period, while RIE-P and RIE-AS cells showed a gradual decline in cell number. RIE-AS cells detached completely from ECM within 72 hr of plating, and RIE-P cells detached within 7 days, whereas all of the RIE-S cells remained attached to the cell culture plate for 2 weeks. The results with RIE-P and RIE-AS cells concur with those reported using IEC-6 cells (Carroll et al., 1988). Our results suggested that the cells expressing COX-2 can attach to basement membrane and survive on it, whereas cells in which COX-2 expression is undetectable (RIE-AS) are unable to do so. This clearly demonstrated that the extracellular adhesion pathway was altered by COX-2 overexpression and suggested that cell survival was affected as well.

COX-2 Expression Increases Resistance to Apoptosis in RIE Cells

In light of the above results, we investigated the effect of COX-2 overexpression on apoptosis using two independent techniques: analysis of DNA fragmentation and staining with a DNA-specific dye (Hoechst 33258). Analysis of the RIE-AS cells revealed oligonucleosomal DNA frag-

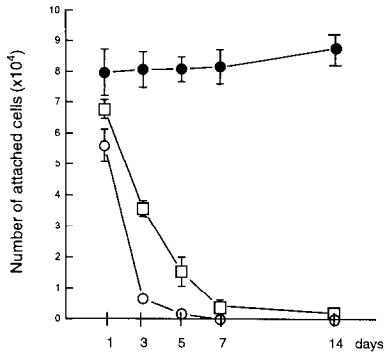

Figure 3. Effect of COX-2 Overexpression on Cell Kinetics of Binding to Matrigel-Coated Plates

RIE-S cells (closed circles), RIE-P (open squares), and RIE-AS (open circles) cells (4 × 10^5) were plated into 24-well plates coated with Matrigel. At various intervals, the cells were washed with PBS, and dipase was added to the cell culture plates for 1 hr. The cells were then resuspended, and cell counts were determined using a hemocytometer.

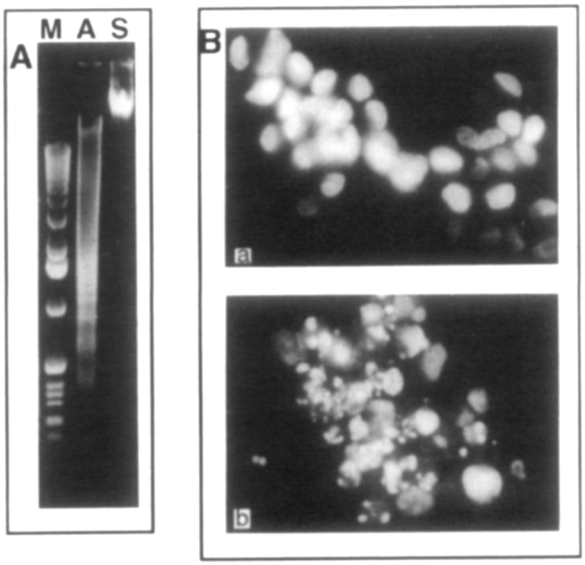

Figure 4. Effect of COX-2 Expression on Apoptosis in RIE Cells

(A) Evaluation of apoptosis using DNA laddering. The electrophoretic pattern of DNA isolated from RIE-S and RIE-AS cells is shown. DNA was extracted from the RIE cells cultured on Matrigel for 3 days and analyzed on a 1% agarose gel. Lane M, 1 kb DNA ladder; lane A, RIE-AS cells; lane S, RIE-S cells. Identical results were obtained following 48 hr of treatment with 5 mM sodium butyrate.

(B) Evaluation of apoptosis using fluorochrome staining. RIE cells were cultured on Matrigel for 3 days, fixed in glutaraldehyde, and stained with the DNA-specific fluorochrome bis-benzimide (Hoechst 33258). The results with RIE-S cells are denoted by the letter a, and results with RIE-AS cells are denoted by the letter b. Identical results were observed for cells following treatment with 5 mM sodium butyrate for 48 hr. Total magnification is 71 ×.

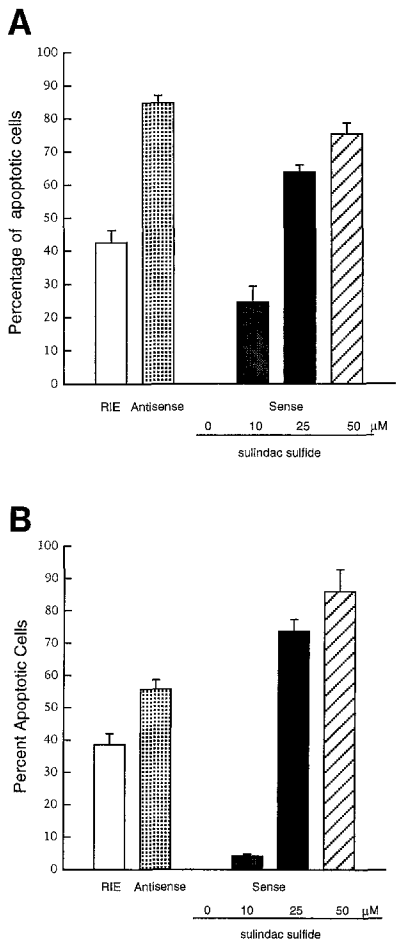

Figure 5. Effect of COX-2 Overexpression on Apoptosis

(A) We plated 1 × 10^6 cells of each group on each 10 cm^2 well coated with Matrigel, and after 24 hr of culture, the wells were washed with PBS. The media was replaced, and the cells were cultured for an additional 3 days. The ratio of apoptotic cells was determined by nuclear staining. Cells with three or more chromatin fragments were considered apoptotic.

(B) Confluent cell cultures were established in 10 cm^2 wells, and the medium was replaced with fresh medium containing 5 mM sodium butyrate. After 4 days, the percentage of apoptotic cells was determined by nuclear staining as described above.

mentation that results in a DNA ladder containing 180 bp fragments when analyzed by neutral agarose gel electrophoresis. DNA laddering such as that demonstrated in Figure 4A was routinely observed within 24 hr following plating of RIE-AS and RIE-P cells on ECM but was not observed for RIE-S cells. Plating of RIE-AS cells on ECM also resulted in the appearance of typical morphological changes of apoptosis upon staining the cells with the DNA-specific fluorochrome bis-benzimide trihydrochloride (Hoechst 33258), as demonstrated in Figure 4B. These changes included condensation of chromatin, its compaction along the periphery of the nucleus, and segmentation of the nucleus.

Other intestinal epithelial cell lines have been reported to undergo differentiation and apoptosis when treated with sodium butyrate (Hague et al., 1993), so the effect of sodium butyrate on RIE cells in culture was examined. RIE cells cultured in medium containing sodium butyrate underwent apoptosis within 3 days in a distinct and reproducible fashion. As shown in Figures 5A and 5B, a significant number of RIE-P and RIE-AS cells cultured under these conditions became apoptotic. However, we observed very few apoptotic RIE-S cells following butyrate treatment. Addition of a COX inhibitor (sulindac sulfide) allowed apoptosis to occur in RIE-S cells following butyrate treatment or when the cells were plated on Matrigel. These results represent additional evidence that overexpression of COX-2 inhibits apoptosis induced by butyrate treatment or by plating the cells on ECM. Addition of sulindac sulfide at the concentrations used in this study to RIE-S cells alone (without butyrate or ECM) resulted in less than 5% of the cells undergoing apoptosis (data not shown).

COX-2 Expression Affects Key Regulatory Proteins in RIE Cells

Alkaline Phosphatase

To examine the effect of COX-2 expression on a marker of intestinal epithelial differentiation, alkaline phosphatase activity (Carroll et al., 1988; Herz et al., 1981) was evaluated. In vivo, after cells migrate from the undifferentiated crypt compartment to the luminal villus compartment, the

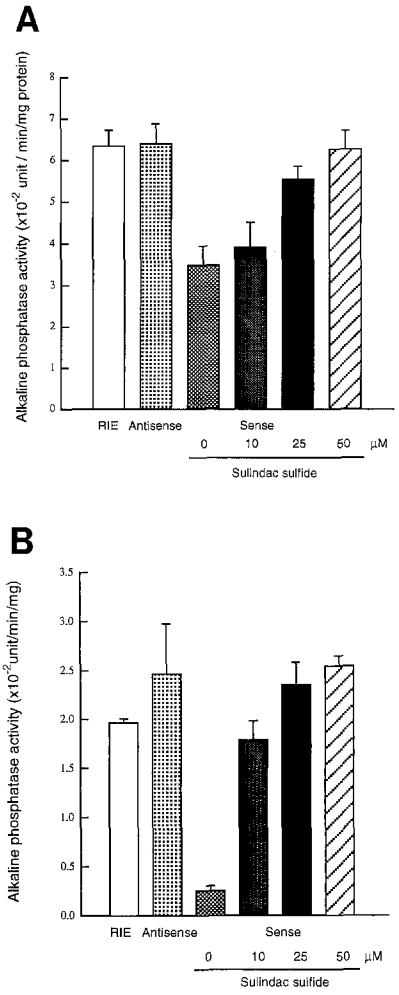

Figure 6. Effect of COX-2 Overexpression on Cellular Differentiation

Cellular differentiation was measured by alkaline phosphatase activity. (A) We plated 1 × 10^6 cells of each group on each 10 cm^2 well coated with Matrigel. After 24 hr, the cells were washed with PBS and cultured for an additional 48 hr. After the culture media was removed, the culture plates were incubated with 0.5 ml of Dipase (Collaborative Research) for 45 min. The cells were washed twice with TBS. The cells were lysed in a 200 μl of TBS containing 0.25% sodium deoxycholate for 30 min at room temperature. The lysate was then centrifuged at 10,000 × g for 15 min at 4°C. Alkaline phosphatase activity was measured at 30°C using the alkaline phosphatase kit purchased from Sigma Chemical Company following the instructions of the manufacturer.

(B) Confluent cells of each group were established in 10 cm^2 wells and cultured in media containing 5 mM sodium butyrate for an additional 4 days. Floating and attached cells were collected (following trypsinization), mixed, and washed twice with PBS. Preparation of cell lysates and measurement of alkaline phosphatase activity were carried out as described above.

expression of alkaline phosphatase increases dramatically (Ferraris et al., 1992; Hodin et al., 1994). Since expression of this enzyme is related to intestinal epithelial differentiation, we measured alkaline phosphatase activity in various RIE cell lines to obtain information about the state of differentiation of these cells. RIE-AS and RIE-P cells were cultured on dishes coated with Matrigel for 3 days or on plastic and then treated with sodium butyrate.

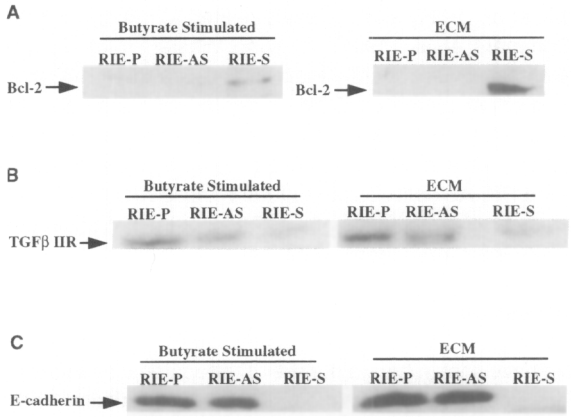

Figure 7. Western Blot Analysis for BCL2, E-Cadherin, and TGFβ2 Receptor Protein Levels

RIE-P, RIE-S, and RIE-AS cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). The cells were plated on plastic dishes and treated with sodium butyrate or grown on Matrigel. The cells were harvested and lysed in RIPA buffer. Protein (100 μg) of the cell lysate samples was separated on 12.5% SDS–polyacrylamide gels. Cellular expression of BCL2 (A) and TGFβ2 receptor (B) were detected using a commercially available rabbit polyclonal antibody, and E-cadherin (C) was detected using a mouse IgG2α monoclonal antibody by Western blotting procedures (ECL system; Amersham).

The RIE-AS cells, which have undetectable COX levels, showed high levels of alkaline phosphatase activity compared with RIE-S cells (Figures 6A and 6B). In the presence of increasing sulindac sulfide concentrations, RIE-S cells showed increased alkaline phosphatase activity. The effect was more pronounced when the cells were plated on plastic culture dishes (Figure 6B) and treated with sodium butyrate compared with the cells plated on Matrigel-coated plates. These results also demonstrate that the effect of COX-2 overexpression can be reversed by adding an inhibitor of the enzyme, such as sulindac sulfide.

BCL2, TGFβ2 Receptor, and E-Cadherin

BCL2 expression has been linked to inhibition of apoptosis in several different cell types (Hague et al., 1994; Korsmeyer, 1992). Because the RIE-S cells were resistant to apoptosis, we measured BCL2 protein levels to see whether they correlated with this phenotype. As shown in Figure 7A, BCL2 levels were elevated in RIE-S cells plated on plastic and treated with sodium butryate, but were even higher in RIE-S cells plated on Matrigel. BCL2 expression was undetectable in RIE-P or RIE-AS cells under either condition tested. TGFβ2 receptors and E-cadherin have both been implicated in colorectal tumorigenesis. TGFβ2 receptors were found to be inactivated in a subset of colon cancer cell lines that exhibited microsatellite instability (Markowitz et al., 1995). Therefore, we measured TGFβ2 receptor protein levels in RIE-S, RIE-AS, and RIE-P cells. We found that TGFβ2 receptor levels were much lower in the RIE-S cells either treated with butyrate or plated on ECM (Figure 7B). Down-regulation of E-cadherin has been reported to occur in a variety of solid tumors (Mayer et al., 1993; Shimoyama and Hirohashi, 1991a, 1991b; Shio-

zaki et al., 1991) and is closely related to tumor invasion (Sommers et al., 1991). Utilizing Western blotting, we quantitated E-cadherin levels in RIE-S and RIE-AS cell lines either plated on ECM or treated with butyrate. As seen in Figure 7B, we found that E-cadherin protein was undetectable in the RIE-S cells but was highly expressed in the RIE-AS cells. Taken together, these results demonstrate biochemical changes in RIE-S cells that could enhance their tumorigenic potential.

Discussion

We have shown here a number of phenotypic changes in RIE-S cells that overexpress COX-2. In particular, they attach to ECM components (e.g., laminin) with greater avidity than cells that do not express this enzyme, express lower levels of TGFβ2 receptor and E-cadherin, resist undergoing apoptosis when stimulated to do so, and express high levels of BCL2 protein. Each of these changes could enhance the tumorigenic potential of intestinal epithelial cells. Since COX-2 expression levels increase dramatically in colorectal cancer (Eberhart et al., 1994; Kargman et al., 1995), our findings may relate to the ability of COX inhibitors, such as aspirin and other NSAIDs, to reduce the relative risk of colorectal cancer in humans.

The results of this work demonstrate that COX-2 overexpression may alter intestinal epithelial biology in a number of ways. Several studies suggest that cell–substrate adhesion may play an important role in tumorigenicity, since cells that have been transformed by a virus or with chemical carcinogens demonstrate altered adhesion to ECM compared with nontransformed cells (Behrens et al., 1989; Dedhar and Saulnier, 1990; Plantefaber and Hynes, 1989). Other groups have reported that cellular attachment to laminin had a positive effect on cell proliferation, whereas attachment to fibronectin-inhibited proliferation (Giancotti and Ruoslahti, 1990). Programmed cell death is one of the most important components in maintaining the integrity of intestinal epithelium. Cellular differentiation and proliferative arrest are induced as intestinal stem cells ascend upward on basement membrane along the crypt–villus axis toward the intestinal lumen. When the cells reach the surface of the villi, they become fully differentiated, undergo programmed cell death, and are shed into the lumen. The entire lifetime of an epithelial cell in humans is only 4–5 days. Factors that could prolong cell survival could influence the integrity and function of intestinal mucosa. Additionally, prolonged cell survival and increased adhesion to matrix components could have significant biological consequences and effect the tumorigenic potential of epithelial cells. Recently, the study of apoptosis has become the focus of a number of groups evaluating the molecular basis for the development of colorectal cancer (Hague et al., 1993; Markowitz et al., 1995; Pasricha et al., 1995). Prolonged survival of abnormal cells can favor tumor progression and facilitate the accumulation of sequential genetic mutations that would result in tumor promotion. In the context of intestinal epithelium in vivo, high COX-2 levels would increase the time that abnormal cells (with a 1° mutation) would spend in transit to the villus tip and increase the chance that a second mutation could occur (tumor progression). Our results are consistent with this hypothesis, as they demonstrate that RIE-S cells have prolonged survival under conditions in which apoptosis is normally induced. They also support a protective role of NSAIDs, since following inhibition of COX the cells undergo programmed cell death quite readily.

The effect of COX-2 overexpression on TGFβ2 receptor and E-cadherin expression levels could also play a significant role in tumorigenesis. TGFβ inhibits the growth of epithelial cells (Barnard et al., 1989), and some reports have indicated that loss of this negative regulation contributes to colorectal tumor development (Markowitz et al., 1994). The TGFβ growth inhibitory signal is transduced through both the TGFβ1 and TGFβ2 receptors (Lin and Moustakas, 1994; Lin et al., 1995). It was recently demonstrated (Markowitz et al., 1995) that TGFβ2 receptor transcripts were undetectable or present at markedly reduced amounts in some human colon cancer cell lines, although TGFβ1 receptor transcripts were detected in all samples. Reduced TGFβ2 receptor expression, by inducing the escape of cells from TGFβ-mediated growth control, would also increase the probability that cells expressing high COX-2 levels would have prolonged survival and at risk for tumor progression.

Down-regulation of E-cadherin has been reported to occur in multiple solid tumors (Mayer et al., 1993; Shimoyama and Hirohashi, 1991a, 1991b; Shiozaki et al., 1991) and is closely related to invasion (Sommers et al., 1991). Additional evidence supports the idea that E-cadherin may act as an invasion-suppressor gene (Behrens et al., 1989; Frixen et al., 1991; Oka et al., 1993). For tumor formation, local invasion is thought to be one of the most important factors. Moreover, some reports have shown that more than 80% of poorly differentiated tumors lack expression of E-cadherin. These results suggest that the presence of E-cadherin is important in cell differentiation and that down-regulation of E-cadherin expression is associated with local invasion of tumor cells.

Colorectal cancer is the second leading cause of death from cancer in the United States. In early stages of the disease a cure is possible, but unfortunately, these malignancies have often grown beyond the large intestine by the time they are detected and our current treatment regimens are not very effective. Work carried out by several groups over the last decade has focused on the molecular basis of colorectal cancer, and tremendous progress has been made. Clinical investigators have observed a 40%–50% reduction in the relative risk of colorectal cancer in humans who take NSAIDs (like aspirin) on a regular basis. One of the molecular targets for NSAIDs is COX. Our work presented here demonstrates that when intestinal epithelial cells overexpress one of the COX isoforms (COX-2), they adhere more avidly to ECM proteins and are resistant to apoptosis. Both of these phenotypic changes are reversed by the addition of a COX inhibitor (sulindac sulfide). These results may provide some insight regarding the molecular basis for the chemoprotective effects of NSAIDs.

Experimental Procedures

Cell Culture

RIE-1 cells were a gift from K. D. Brown (Cambridge Research Station, Babraham, Cambridge, England). Cells were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal bovine serum (lot number 11151032; Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100,000 U/l penicillin G, and 100,000 μg/l streptomycin sulfate.

Stable Transfection

A 2.1 kb SmaI–EcoRV fragment containing the open reading frame for a polypeptide of 604 amino acids of rat COX-2 was isolated and blunt end cloned into the SmaI site of the eukaryotic expression vector, pCB7 (a gift of R. Coffey, Vanderbilt University). In this vector, transcription of the cDNA is controlled by the CMV promoter. This vector also contains a bacterial hygromycin resistance gene expression cassette that allows for selection in hygromycin B. Sense- and antisense-oriented expression vectors were prepared. These expression vectors were transfected into a nontransformed RIE cell line (RIE-1) using a Lipofectin (GIBCO–BRL) transfection method (DuBois et al., 1994b), and the cells were selected in medium containing hygromycin B (225 U/ml). Total RNA was prepared from the cells transfected with sense-oriented expression vector and evaluated using Northern slot blot analysis. Five clones (S10, S99, S123, S132, and S146) expressing the highest level of *COX-2* RNA were identified from 225 initial clones and expanded. We characterized all five clones and found that they all exhibited the identical phenotypic and biochemical alterations. Therefore, the results of our studies using the RIE-S10 clone are presented here. The RIE cells transfected with antisense expression vector (RIE-AS) were evaluated in a similar manner. It is important to note from previous studies that RIE cells have undetectable levels of *COX-1* mRNA and protein (DuBois et al., 1994a).

Western Blotting

To confirm the expression of COX-2 in sense-transfected cells, Western blot analysis and COX enzyme assays were done. RIE cells transfected with the sense- and antisense-oriented *COX-2* cDNA were grown in DMEM supplemented with 10% fetal bovine serum (FBS). The cells were harvested and lysed in RIPA buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 ng/ml PMSF, 66 ng/ml aprotinin). Samples containing 100 μg of cell lysate protein were separated on 12.5% SDS–polyacrylamide gels. Total protein was assayed using protein assay reagent (Pierce, catalog number 23225). COX-2 protein levels were detected by using a rabbit polyclonal antibody (DuBois et al., 1994a) and by using standard detection techniques (ECL system; Amersham).

For Western blotting of BCL2, the cells were cultured on Matrigel (Collaborative Biomedical Products, catalog number 40230) or in medium containing 5 mM sodium butyrate (Sigma Chemical Company) for 24 hr, harvested, and lysed in RIPA buffer. Samples containing 100 μg of protein were separated on 15% SDS–polyacrylamide gels. BCL2 protein levels were determined using a rabbit polyclonal antibody (Santa Cruz, catalog number SC-492) and standard detection techniques (ECL system; Amersham). For TGFβ2 receptor and E-cadherin Western blot analysis, cell lysates containing 100 μg of protein were separated on 7.5% SDS–polyacrylamide gels. TGFβ2 receptor expression was detected by using a rabbit polyclonal antibody (Santa Cruz, catalog number SC-400) that has been carefully evaluated and does not cross-react with TGFβ1 receptor. E-cadherin expression was detected using a mouse monoclonal anti-human E-cadherin antibody (Transduction Laboratories, catalog number C20820).

COX Assays

For COX enzyme assays, the cells were harvested and resuspended in phosphate-buffered saline (PBS) containing 25 μM [^{14}C]arachidonate (Dupont–New England Nuclear) for 15 min at 25°C with constant mixing. Various concentrations of sulindac sulfide were added in selected experiments. The organic soluble reaction products were extracted into ethyl acetate and separated by reverse-phase HPLC using a μBondapak C_{18} column (Waters) utilizing an acetonitrile–water gradient. The radioactive metabolites in the eluent were monitored by an on-line scintillation counter (DuBois et al., 1994b). The COX activity was calculated by arachidonic acid conversion rate (nmole/mg protein).

Cell Attachment Studies

Cells (4×10^5) of each group were plated into 2 cm^2 wells coated with Matrigel, laminin (Collaborative Biomedical Products, catalog number 40232), or fibronectin (Collaborative Biomedical Products). After the plates were incubated at 37°C for 45 min, the wells were washed with PBS (9.1 mM dibasic sodium phosphate, 1.7 mM monobasic sodium phosphate, 150 mM NaCl [pH 7.4]) to remove unattached cells. Attached cells were incubated for 1 hr in dipase (Collaborative Biomedical Products, catalog number 40235) and resuspended, and cell counts were determined using a hemocytometer.

For cell culture on Matrigel, 4×10^5 of RIE-S, RIE-P, and RIE-AS transfected cells were plated into 24-well plates coated with Matrigel. At various time intervals, cells were washed with PBS and incubated in dipase for 1 hr. The cells were resuspended, and cell counts were determined using a hemocytometer.

Alkaline Phosphatase Activity

Alkaline phosphatase activities of the cells were determined as a marker of cell differentiation.

Culture on Matrigel

We plated 1×10^6 cells of each group on each 10 cm^2 well coated with Matrigel. After 24 hr, the cells were washed with PBS and cultured for an additional 48 hr in the presence of various concentrations of sulindac sulfide (with media changes every 12 hr). Following this incubation period, the culture media were removed, and the culture plates were incubated with 0.5 ml of Dipase (Collaborative Research) for 45 min. After the cells were washed twice with TBS (10 mM Tris–HCl, 150 mM NaCl), they were lysed in a 200 μl of TBS containing 0.25% sodium deoxycholate for 30 min at room temperature. The lysate was then centrifuged at 10,000 × g for 15 min at 4°C. Alkaline phosphatase activity was measured at 30°C using a commercial alkaline phosphatase kit (Sigma Chemical Company, catalog number 245-10).

Culture in Media Containing Sodium Butyric Acid

Confluent cells of each group were established in 10 cm^2 wells and cultured in the media containing 5 mM sodium butyrate and various concentrations of sulindac sulfide for an additional 96 hr (with media changes every 12 hr). The cells were washed with TBS prior to harvest.

Apoptosis

Culture on Matrigel

We plated 1×10^6 cells of each group (RIE-P, RIE-AS, and RIE-S) on 10 cm^2 wells coated with Matrigel, and after 24 hr of culture, the cells were washed with PBS. The media was replaced, and the cells were cultured for an additional 72 hr and various concentrations of sulindac sulfide were added (with media changes every 12 hr).

Culture in Media Containing Sodium Butyric Acid

Confluent cell cultures were established in 10 cm^2 wells, and the medium was replaced with fresh medium containing 5 mM of sodium butyrate and various concentrations of sulindac sulfide every 12 hr. After 96 hr, the percentage of floating cells was calculated.

DNA was extracted from RIE cells cultured on Matrigel or treated with 5 mM sodium butyrate and analyzed on a 1% agarose gel. Nuclei of floating cells and attached cells were stained with a DNA-specific fluorochrome bis-benzimide trihydrochloride (Hoechst 33258; Sigma Chemical Company). After fixing cells with glutaraldehyde, cells were stained in 167 μM bis-benzimide and observed under fluorescent microscopy. Cells with three or more chromatin fragments were considered apoptotic.

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