Sulindac Sulfone Inhibits K-ras-dependent Cyclooxygenase-2 Expression in Human Colon Cancer Cells

Michele T. Taylor, Kathryn R. Lawson, Natalia A. Ignatenko, Sarah E. Marek, David E. Stringer, Beth A. Skovran, and Eugene W. Gerner


ABSTRACT
Both the sulfide and sulfone metabolites of sulindac, a nonsteroidal anti-inflammatory drug, display anticarcinogenic effects in experimental models. Sulindac sulfide inhibits cyclooxygenase (COX) enzyme activities and has been reported to suppress ras-dependent signaling. However, the mechanisms by which sulindac sulfone suppresses cancer growth are not as defined. We studied the effects of these sulindac metabolites in human colon cancer-derived Caco-2 cells that have been transfected with an activated K-ras oncogene. Stable transfected clones expressed high levels of COX-2 mRNA and protein, compared with parental cells. K-ras-transfected cells formed tumors more quickly when injected into severe combined immunodeficiency disease mice than parental cells, and this tumorigenesis was suppressed by treatment with sulindac. Sulindac sulfone inhibited COX-2 protein expression, which resulted in a decrease in prostaglandin synthase E2 production. Sulindac sulfide had little effect on COX-2 in this model, but did suppress prostaglandin synthase E2 production, presumably by inhibiting COX enzyme activity. These data indicate that the sulfide and sulfone derivatives of sulindac exert COX-dependent effects by distinct mechanisms.

INTRODUCTION
The COX pathway has been under intense investigation as a target for the treatment and prevention of colorectal cancer. Several studies have reported a 40–50% decrease in mortality from colorectal cancer with prolonged use of NSAIDs1–4. Sulindac, an inhibitor of COX-1 and COX-2, has been reported to reduce the size and number of colorectal tumors in FAP patients as well as in animal models of FAP (5). Sulindac is metabolized into sulfone and sulfide derivatives. Both derivatives are known to inhibit cell growth by the induction of apoptosis (6). The sulfide metabolite has been shown to have COX enzyme inhibitory activity, whereas the sulfone metabolite lacks this effect (7, 8). Recent studies have shown that the sulfone can prevent tumor formation in rodent models of chemical carcinogenesis without inhibiting COX enzyme activity or lowering prostaglandin levels (9, 10). Sulindac, which is now referred to as exisulind (Aptosyn) is currently being developed for the treatment of FAP patients as well as for other cancer chemopreventive and therapeutic indications.

The first evidence linking an activated ras oncogene to up-regulation of COX-2 was one in which an activated H-ras gene was transfected into rat intestinal epithelial cells (11). Treatment with the COX-2 selective inhibitor, SC58125, suppressed growth in these cells and induced apoptosis. An activated Ha-ras transfected into rat-1 fibroblasts caused an increase in transcription and in the half-life of the COX-2 message, which appeared to be modulated by the MAPK pathway (12). Selective inhibition of the MAPK kinase by PD98059 caused a delay in COX-2 induction at both the mRNA and the protein levels. This suggests that the MAPK signaling cascade may mediate COX-2 expression.

Sulindac sulfide has been shown previously to bind ras to inhibit the recruitment of raf to the plasma membrane and thus directly inhibit ras signaling (13). In addition, sulindac sulfone and certain NSAIDs (i.e., piroxicam and sulindac) have been shown previously to have a proportionately greater inhibitory effect on carcinomas harboring mutations in either K- or H-ras (14, 15). To determine whether sulindac sulfone can affect ras-dependent signaling, we investigated the effect of this drug on the K-ras-dependent expression of COX-2 in a human colon cancer-derived cell line.

MATERIALS AND METHODS
Cell Culture and Transfections. Caco-2 cells were maintained in MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. All cell culture supplies were obtained from Life Technologies, Inc., Rockville, MD. K-ras cDNA was purchased from American Type Culture Collection (Rockville, MD) and ligated into the multiple cloning site of a pCDNA3 mammalian expression vector (Invitrogen Corp., Carlsbad, CA) containing a cytomegalovirus promoter and a neomycin resistance gene. The pCDNA3-K-ras plasmid was transfected into Caco-2 cells using the calcium phosphate transfection technique as described in the literature (16). The K-ras-activated clones were maintained under selection with 350 μg/ml G418. Sulindac (the sulfone form), sulindac sulfide, and sulindac sulfone were purchased from ICN Biochemicals, Inc., Aurora, OH, and obtained as a generous gift from G. A. Piazza (Cell Pathways, Inc., Horsham, PA). Previous work from our laboratory indicated that the dose to reduce colony formation by 50% (IC50 dose) in both parental and K-ras-transfected clones was 120 μM for sulindac sulfide, 600 μM for sulindac sulfone, and 400 μM for sulindac sulfone (17).

PCR Assay for K-ras Mutations. Caco-2 cells and K-ras-activated clones were lysed in buffer containing 100 mM NaCl, 10 mM Tris-HCl (pH 8), 25 mM EDTA, 0.5% SDS, and 100 μg/ml proteinase K and incubated at 37°C for 16 h. DNA was isolated with two extractions in phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated on ice in 3 M sodium acetate and 100% ethanol. The pellet was washed in 70% ethanol and further purified in 100 μg/ml RNase, 0.5% SDS, and 100 μg/ml proteinase K. Another phenol:chloroform:isoamyl alcohol extraction was performed, the DNA pellet was precipitated with ethanol and then resuspended in TE (10 mM Tris·HCl·1 mM EDTA) buffer. DNA concentration was determined by UV spectrophotometric methods. PCR was performed with specific primers (Life Technologies, Inc.) to create a BstNI restriction site at codon 12 of the human K-ras gene. The upstream primer sequence used was 5′: AAA CTT GTG GTA GTT GGA CCT; the downstream primer was 5′: TTG TTG CAT ATT CGT CC. Mutation at codon 12 of K-ras alters the BstNI site, preventing restriction enzyme cleavage. PCR products were run out on a 4% NuSieve:agarose (3:1) gel stained with ethidium bromide.

Immunoblotting. Subconfluent cells were lysed on ice in radioimmunoprecipitation assay buffer (PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 30 μg/ml aprotinin, 100 mM sodium orthovanadate, and 10 mg/ml phenylmethylsulfonyl fluoride) and electrophoresed on a 10% SDS-PAGE gel. Proteins were transferred to nitrocellulose membrane. Membranes were blocked in Blotto A, probed with the appropriate antibodies, and developed by the enhanced chemiluminescence system (Amersham, Arlington Heights, IL).
COX-1 and COX-2 antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA, and used at 1:250 and 1:5000 dilutions, respectively. All Western blots were repeated three times and a representative blot was chosen for presentation.

**Northern Blotting.** RNA was isolated from frozen cell pellets using Trizol:chloroform (5:1; Life Technologies, Inc.) extractions. RNA was further purified in isopropanol and washed in 75% ethanol. RNA was run out on a 1% agarose/formaldehyde gel in MOPS [3-(N-morpholino)propanesulfonic acid] buffer and transferred to nylon. The COX-2 cDNA probe (Oxford Biomedical Research, Inc., Oxford, MI) was labeled using the KAT RadPrime DNA labeling system (Life Technologies, Inc.) and [α-32P]-dCTP. The probe was purified with G-50 Sepharose columns (Boehinger Mannheim, Indianapolis, IND) and hybridized with a scintillation counter. The membrane was hybridized to the probe overnight at 42°C and then washed three times (2× SSC-0.1% SDS for 5 min at room temperature, then for 20 min at room temperature, and then 0.5× SSC-0.1% SDS for 30 min at 65°C). Membranes were prehybridized again and then hybridized to glyceraldehyde-3-phosphate dehydrogenase (0.75 kb PstI-XbaI fragment) as a loading control. Autoradiograms were quantitated by densitometric analysis (ImageQuant; Molecular Dynamics, Sunnyvale, CA).

**PGE2 Production.** Cells were seeded 10⁴/well on 96-well plates and treated with drug or vehicle 24 h later. Serum-free medium was supplemented with 15 μM arachidonic acid (Sigma, St. Louis, MO) after 24 h of drug treatment for 1 h prior to medium collection for the PGE₂ kit (Amersham, Arlington Heights, IL).

**Animal Model.** Caco-2- or K-ras-activated Caco-2 cells (clone 60) were injected subdermally into four areas of the flank of SCID mice at 1×10⁶ cells/injection. There were three to four mice/group. The mice were fed 167 parts/million sulindac in AIN93G diet (Teklad, Indianapolis, IN). The injection was repeated twice a week. The animals were sacrificed at 100 days because of tumor burden.

**Statistics.** Assessment of statistical differences for Figs. 3 and 4 were determined by ANOVA. A P <0.05 was considered statistically significant.

**RESULTS**

**Characterization of K-ras-activated Clones.** Caco-2 cells were used for stable transfection of p21Val12 K-ras because they express normal K-ras (15). To better elucidate K-ras overexpression, we used a PCR-RFLP technique to determine whether the transfectants overexpressed the normal or mutant alleles. We introduced a BstNI restriction enzyme cut site using specific primers and then cut the PCR product; the enzyme cuts if the cells are expressing the normal allele, but it will not cut if the cells are expressing the mutant allele. The data shows that the clones with the highest expression (transfected clone numbers 60, 66, and 96) displayed both normal and mutant alleles, whereas the parental cell line expressed only normal K-ras (Fig. 1A). This shows that the transfected cells do indeed express the mutant K-ras gene but does not provide information regarding the level of the normal or the mutant allele being expressed. Because transfection with H-ras changes the growth characteristics of Caco-2 cells (18), we examined the growth of the K-ras transfectants and compared them with the parental line. After 12 days in culture, there was no significant difference in growth kinetics between the parental cell line and the K-ras-activated clones (Fig. 1B). This may indicate that both normal and mutant alleles are being overexpressed simultaneously in the transfectants.

**COX Expression in K-ras-activated Caco-2 Cells.** We measured COX-2 protein and RNA levels in the K-ras-activated clones and in the parental Caco-2 cell line. The level of COX-2 protein was increased in the K-ras-activated cells relative to the parental cell line,
mRNA and protein, we expected to observe an increase in PGE\textsubscript{2} production. Western blot analysis of COX-2 in Caco-2 cells and K-ras transfectants treated with 600 μM sulfone. The use was DMSO. K-ras-activated Caco-2 cells exhibited an elevation in COX-2 synthesis over the parental cells (Fig. 3A). PGE\textsubscript{2} production was next measured in cells treated for 24 h with sulindac and its sulfide and sulfone metabolites at doses previously established to inhibit Caco-2 colony formation by 50%. Treatment with 400 μM sulindac sulfoxide significantly inhibited PGE\textsubscript{2} production in K-ras-activated cells (data not shown). Sulindac sulfone at 600 μM and sulfide at 120 μM displayed similar inhibitory effects on prostaglandin synthesis (Fig. 3C). In addition, sulfone significantly inhibited COX-2 protein (and mRNA, data not shown), whereas the sulfide at its IC\textsubscript{50} dose did not affect protein levels (Fig. 3A). This finding suggests that the sulfone can indirectly affect prostaglandin synthesis by affecting COX-2 protein expression. By contrast, an IC\textsubscript{50} dose of sulindac sulfide suppresses PGE\textsubscript{2} levels, presumably by inhibiting COX enzyme activity, but does not affect COX-2 protein expression. This is the first demonstration that sulindac sulfone can exert COX-dependent effects on cell behaviors, specifically by suppressing K-ras-dependent signaling of COX-2 protein expression.

Sulindac inhibited tumor formation in SCID mice injected with K-ras-activated Caco-2 cells (Fig. 4). Caco-2 cells and the K-ras-activated transfectants showed no difference in growth characteristics \textit{in vitro} (Fig. 1B) but displayed differences in tumor formation when injected in immune-compromised mice. K-ras activation caused an increase in the rate of tumor formation over the nontransfected cell line, and sulindac greatly inhibited this tumorigenesis. Measurable tumors became apparent in untreated mice after 35 days, whereas sulindac-treated mice did not present with tumors until after day 60. By day 100, sulindac had prevented tumor growth by 60% compared with the control group.

**DISCUSSION**

The data presented here indicate that sulindac and its sulfide and sulfone metabolites inhibit tumorigenesis in colon-derived cells by at least two distinct mechanisms. One mechanism involves the suppression of prostaglandin synthesis by COX-1 and/or COX-2 enzyme activity (8). The second mechanism, shown here for the sulfone metabolite, involves inhibition of the K-ras-dependent signaling of genes affecting tumorigenesis. Sulindac sulfone has been shown to affect the signaling pathways influencing the expression of genes other than COX-2, as shown here. Yamamoto et al. (19) have reported that this drug inhibits NF-κB activation in colon cancer and other cell types. We have observed an increase in promoter activity in the polyamine catabolic enzyme, spermidine/spermine N\textsuperscript{2}-acetyltransferase when K-ras-activated Caco-2 cells are treated with sulfone.\textsuperscript{4} These observations suggest that sulfone is acting to modulate the K-ras-dependent signaling of a variety of genes influencing cellular processes. Recently, it has been reported that sulindac sulfone affects β-catenin signaling by inhibition of cyclic GMP-dependent phosphodiesterases and activation of protein kinase G (20).

NSAIDs are known to exert their effects by COX-dependent and -independent mechanisms (21). COX-dependent mechanisms would include those situations in which NSAIDs interact directly with COX proteins to inhibit enzyme activity and those in which agents, such as sulindac sulfone, suppress COX protein expression. This latter mechanism does not involve direct enzyme/inhibitor interactions, but only requires that formation of the target enzyme is prevented. COX-independent mechanisms could involve inhibition of signaling pathways affecting genes other than COX-1 or COX-2.

In the studies reported here, we compared the effects of IC\textsubscript{50} concentrations of the sulfide (120 μM) and sulfone (600 μM) metabolites of sulindac on the K-ras-dependent expression of COX-2. The increased potency of sulindac sulfide may reflect the ability of this metabolite to both suppress COX enzyme activity and inhibit signaling, whereas sulindac sulfone only inhibits signaling. This suggests...
that higher concentrations of sulindac sulfide may also inhibit the signaling of COX-2 protein expression. Hermann et al. (13) have reported that sulindac sulfide can bind to ras in cell-free studies. Future studies will address whether the sulfone directly binds to ras in cells.

The reduction in PGE2 in the sulindac-treated, K-ras-activated Caco-2 cells may be attributable to its effects on COX-1 or COX-2 or both. The distinction cannot be made by measuring prostaglandin levels. We hypothesize that sulindac sulfone is acting to reduce prostaglandin levels in our Caco-2 model by a K-ras-dependent mechanism acting selectively on COX-2, compared with COX-1. We favor this interpretation, because we observed no change in COX-1 protein levels in the K-ras-activated cells (Fig. 2A), and sulindac sulfone does not appear to affect COX-1 protein levels measured by Western blot (data not shown).

The data presented here provide additional evidence for the role of COX-2 expression in colon carcinogenesis, where up to 50% of large adenomas and adenocarcinomas contain activating mutations in K-ras (22). We showed that an activated K-ras oncogene leads to an up-regulation of COX-2 expression in human adenocarcinoma cells. Previous work has shown that activated H-ras leads to up-regulation of COX-2 in rodent cells (12). However, H-ras is not expressed in human colonic epithelium, whereas K-ras mutations occur with high frequency in colorectal cancers (~50%) and are found in nearly 100% of pancreatic cancers (22–24). K-ras has been shown to be the only ras family member to be essential for development in mice (25). Thus it is possible that, in humans, K-ras may have functions unique from H-ras, as has been shown for mice (25).

This study describes a novel COX-dependent effect of the NSAID sulindac, namely the inhibition of K-ras-dependent signaling of COX-2 protein expression by the sulfone metabolite of this drug. These data demonstrate that sulindac can inhibit K-ras-dependent colon tumorigenesis in this Caco-2 cell model system. The mechanisms responsible for suppression of tumor formation may involve both the direct inhibition of COX enzyme activity, by sulindac sulfide, and the suppression of signaling pathways, by sulindac sulfone, affecting the expression of genes which are required for tumor growth.

ACKNOWLEDGMENTS

The authors thank Dr. Gary Piazza for his thoughtful review of the manuscript.

REFERENCES

Sulindac Sulfone Inhibits K-ras-dependent Cyclooxygenase-2 Expression in Human Colon Cancer Cells


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/60/23/6607

Cited articles
This article cites 22 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/60/23/6607.full.html#ref-list-1

Citing articles
This article has been cited by 9 HighWire-hosted articles. Access the articles at:
/content/60/23/6607.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.