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# Effects of Nonsteroidal Anti-inflammatory Drugs on Proliferation and on Induction of Apoptosis in Colon Cancer Cells by a Prostaglandin-Independent Pathway

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**ABSTRACT.** Nonsteroidal anti-inflammatory drugs (NSAIDs) decrease the incidence of and mortality from colon cancer. We observed that NSAIDs inhibit the proliferation rate, alter the cell cycle distribution, and induce apoptosis in colon cancer cell lines. We evaluated whether the inhibition by NSAIDs of prostaglandin (PG) synthesis is required for their effects on colon cancer cells by studying two human colon cancer cell lines: HCT-15 and HT-29. HCT-15, which lacks cyclooxygenase transcripts, does not produce PGs even when exogenously stimulated, whereas HT-29 produces $PGE_2$, $PGF_{2\alpha}$, and $PGI_2$. HCT-15 and HT-29 cells, when treated for up to 72 hr with 200 μM sulindac sulfide (an active metabolite of sulindac) or 900 μM piroxicam, showed changes in proliferation, cell cycle phase distribution, and apoptosis. Treatment with $PGE_2$, $PGF_{2\alpha}$, and $PGI_2$, following a variety of protocols, and at concentrations between $10^{-6}$ and $10^{-11}$ M, failed to reverse the effects of NSAIDs on these three parameters of cell growth. We concluded that NSAIDs inhibit the proliferation rate of the two colon cancer cell lines independent of their ability to inhibit PG synthesis. Thus, alternative mechanisms for their activity on tumor cell growth must be entertained. These observations may be relevant to the mechanism of colon tumor inhibition by NSAIDs. BIOCHEM PHARMACOL 52;2:237–245, 1996.

**KEY WORDS.** prostaglandins; NSAIDs; colon cancer; cell proliferation; apoptosis; cell cycle

The appreciation of the role of NSAIDs[‖] in human colon cancer represents an important recent development. Several NSAIDs decrease the incidence of and mortality from colon cancer [1, 2]. Furthermore, the NSAID sulindac is the first pharmacologic agent demonstrated to induce regression of colonic polyps in familial adenomatous polyposis (FAP) [3, 4]. These clinical and epidemiological observations were preceded by animal studies, which showed that NSAIDs such as aspirin [5], indomethacin [6], sulindac [7], and piroxicam [8] reduce the number and size of carcinogen-induced colon tumors. The mechanisms by which NSAIDs exert such profound antitumor effects in the colon remain unclear; elucidation of these mechanisms will be of great importance to our understanding of colonic carcinogenesis.

It is generally accepted that NSAIDs produce their anti-inflammatory effects by inhibiting PG synthesis [9]. However, not all NSAIDs inhibit PG synthesis [10–12], and often much higher doses are required to produce anti-inflammatory effects than to inhibit PG synthesis [13]. Such observations question the conventional conceptualization that NSAIDs act primarily or exclusively by inhibiting PG synthesis. It is of interest that NSAIDs inhibit a variety of membrane-associated processes that are not dependent on the cyclooxygenase pathway [14–16].

We have demonstrated that NSAIDs, including sulindac sulfide, piroxicam, and indomethacin, inhibit the proliferation of colon cancer cells *in vitro* [17, 18]. This was attributed, at least in part, to alterations in the cell cycle distribution of these cells and to induction of apoptosis. We examined whether the effects of NSAIDs on cell proliferation, cell cycle distribution, and apoptosis in colon cancer cell lines are mediated by inhibition of PG synthesis. To evaluate these questions, we used the colon cancer cell lines HCT-15, which does not produce PGs, and HT-29, which produces PGs. This report describes our findings from this work, which indicate that these effects of NSAIDs are independent of PG synthesis.

## MATERIALS AND METHODS

### *Cell Lines*

The human colon adenocarcinoma cell lines HT-29 (ATCC HTB 38) and HCT-15 (ATCC CCL 225) were

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‖ *Abbreviations:* NSAIDs, nonsteroidal anti-inflammatory drugs; PG, prostaglandin; FBS, fetal bovine serum; HBSS, Hanks' buffered salt solution; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-polymerase chain reaction; COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; $TxA_2$, thromboxane $A_2$; and $TxB_2$, thromboxane $B_2$.

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obtained from the American Type Culture Collection (ATCC, Rockville, MD). HT-29 cells were cultured in McCoy's 5A medium and HCT-15 cells in RPMI 1640 (Cellgro, Mediatech, Herndon, VA). These media were supplemented with 10% FBS (Gemini Bioproducts, Inc., Calabasas, CA), non-essential amino acids, penicillin (50 U/mL), and streptomycin (50 μg/mL) (all from Life Technologies, Inc., Gaithersburg, MD). Cells were grown as monolayers in P100 plates and were incubated at 37° in 5% $CO_2$ and 90% relative humidity.

### *Reagents*

Sulindac sulfide (desoxy-sulindac, provided by Merck & Co., Rahway, NJ), piroxicam (Sigma Chemical Co., St. Louis, MO), and PGs (Sigma) were dissolved in DMSO (Fisher Scientific, Fair Lawn, NJ). Nitrogen was passed over the PG solutions after each use to prevent oxidation. The DMSO concentration was adjusted to be equal in all media. $PGE_2$, $PGF_{2\alpha}$, $PGI_2$, arachidonic acid, A23187, and mellitin (all from Sigma) were dissolved in DMSO and stored at −20°.

### *Eicosanoid Levels*

These were determined directly in the culture media by radioimmunoassay as previously described [19, 20]. Samples of culture media were centrifuged at 500 *g* at 4° to pellet floating cells, and the supernatant was frozen at −20° until the levels of eicosanoids were determined.

### *Cell Proliferation and Cell Cycle Analysis*

Single cell suspensions were obtained from monolayers of cells as follows. First, cells were washed with PBS pH 7.2, supplemented with 1% BSA (PBS/BSA), and then incubated with Trypsin EDTA (Life Technologies, Inc., Grand Island, NY). Then cells were collected from the dishes in the presence of PBS/BSA. Cells floating in the culture medium were harvested by low-speed centrifugation, and pooled with the adherent cell fraction. The cells were washed and resuspended in 1 mL of PBS/BSA. Aliquots of cells were counted using a hemacytometer.

For cell cycle analysis, cells were fixed with ice-cold 70% ethanol, incubated at −20° for a minimum of 30 min, then treated with 0.1% NP-40 for 5 min on ice, and washed with PBS/BSA. Next cells were resuspended in 40 μg/mL propidium iodide (Sigma) and 200 μg/mL RNase type IIA (Sigma) in PBS/BSA and incubated at room temperature for 30 min prior to measuring their DNA content using a Coulter ELITE flow cytometer. The MULTICYCLE AV software program (Phoenix Flow Systems, San Diego, CA) was used to generate DNA content frequency histograms and to assist in data analysis.

### *Apoptosis*

Cells were evaluated for evidence of apoptosis by three different assays: (a) measurement of the DNA content by propidium iodide staining and flow cytometric analysis to detect changes associated with DNA fragmentation occurring with apoptosis [21, 22]; (b) acridine orange staining to identify cellular morphologic changes characteristic of apoptosis [23]; and (c) agarose gel electrophoresis of genomic DNA to detect the DNA degradation associated with apoptosis [24].

ACRIDINE ORANGE STAINING. For fluorescence microscopy, 50,000 freshly harvested cells from control or drug-treated dishes were applied to glass slides by centrifugation at 700 *g* in a cytospin well (Cytospin 2, Shandon Inc., Pittsburgh, PA) for 5 min. The slides were fixed by immersion in acetic acid:ethanol (1:9, v/v) for at least 20 min. Upon drying, the slides were washed with HBSS for 1 min, treated with ice-cold 0.1% Triton X-100/0.08 N HCl/0.15 M NaCl (1 min), and stained with 20 μM acridine orange (Sigma) in a phosphate-citric acid buffer (pH 6) containing 1 mM EDTA and 0.15 M NaCl. Following another wash with HBSS, the slides were covered with cover slips, and cells were examined under fluorescence microscopy and photographed (Nikon Inc., Labophot, Melville, NY). The morphologic criteria used to quantify the fraction of apoptotic cells on these slides included the presence of (a) cytoplasmic and nuclear shrinkage; (b) chromatin condensation; and (c) cytoplasmic blebbing with maintenance of the integrity of the cell membrane (zeiosis) [25]. The percentage of apoptotic cells among all of the cells present on the slide was determined by counting ten randomly selected high-power fields.

DNA DEGRADATION. Cells were plated at a density of $10 \times 10^6$ cells/dish and treated with control or drug-supplemented medium for 48 hr. Genomic DNA was obtained from $4.5 \times 10^{-6}$ HCT-15 cells by lysis in 0.04 M Tris-acetate/1 mM EDTA/0.25% NP-40. These extracts were digested with 100 μg/mL boiled RNase IIA (Sigma) for 1 hr at 37° followed by treatment with proteinase K (Boehringer–Mannheim, Indianapolis, IN) at a concentration of 1 mg/mL at 37° for 18 hr. Aliquots of DNA from the equivalent of $1 \times 10^6$ cells were mixed with sample buffer (final concentration: 0.025% bromophenol blue ± 0.025% xylene cyanol/3.0% glycerol) and resolved in 1.8% agarose gels impregnated with ethidium bromide (0.1 μg/mL). DNA was visualized by UV transillumination and photographed using Polaroid 667 film.

### *RT-PCR*

To identify mRNA for COX-1 and COX-2 in HT-29 and HCT-15 cells, exact primers were synthesized based on an analytical RT-PCR procedure developed for human cyclooxygenases [26, 27]. For human COX-1, the primers were 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ (a 24-mer sense oligonucleotide at position 516) and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (a 24-mer antisense oligonucleotide at position 819), giving rise to a 303 bp PCR product [26, 28]. For human COX-2, the prim-

ers were 5′-TTCAAATGAGATTGTGGGAAAATT-GCT-3′ (a 27-mer sense oligonucleotide at position 573) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (a 24-mer antisense oligonucleotide at position 878), giving rise to a 305 bp PCR product. Equal quantities (500 ng) of mRNA from cultured HT-29 and HCT-15 cells were reverse-transcribed and amplified using an RT-PCR kit (Perkin–Elmer Cetus, Norwalk, CT) following the instructions of the manufacturer. PCR was performed for 40 cycles, using a cycling program of 94° for 1 min, 55° for 1 min, and 72° for 1 min in a 2 Precision Scientific GTC-1 Thermal Cycler. As a control, actin mRNA was also reverse-transcribed and amplified, using the following primers: 5′-GTTTGAGACCTTCAACACCCC-3′ (a 21-mer sense oligonucleotide at position 409) and 5′-GTGGCCATCTCCTGCTCGAAGTC-3′ (a 23-mer oligonucleotide at position 727), giving rise to a 318 bp PCR product. PCR was performed for 35 cycles, using a cycling program of 94° for 1 min, 60° for 1 min, and 72° for 1 min. Following the amplification, an aliquot of reaction mixture was fractionated by electrophoresis in a 2% agarose gel.

### *Statistical Methods*

All experiments were repeated at least three times, and similar results were obtained. Unpaired *t*-tests were used for statistical analysis. A value of $P < 0.05$ was considered significant.

## RESULTS

### *PG Production by HCT-15 and HT-29 Colon Adenocarcinoma Cells*

We determined the profile of PG production by the HCT-15 and HT-29 cells. Cells ($1 \times 10^6$) were plated in P100 Petri dishes in the presence of FBS for 24 hr at which time cells were washed three times with prewarmed PBS and once with medium without FBS. Cells were then incubated in culture medium either with or without supplementation with FBS. After another 24 hr, the culture medium was harvested, and the PGs were assayed. As can be seen in Table 1, in the absence of FBS, HCT-15 cells did not produce any PGs, whereas the HT-29 cells produced $PGE_2$ (0.051 ± 0.008 ng/mL; mean ± SEM), $PGF_{2\alpha}$ (0.01 ± 0.007 ng/mL), and $PGI_2$ (0.023 ± 0.011 ng/mL). Neither cell line appeared capable of producing $TxA_2$, which was assayed as its product, $TxB_2$. When either cell line was cultured in the presence of serum, the culture medium of both contained PGs. In the case of HT-29 cells, the increased amounts of PGs appeared to result from the exogenously added $PGE_2$ and $PGF_{2\alpha}$ present in FBS, and also from the endogenous PG production stimulated by the serum. In the case of HCT-15 cells, the $PGE_2$ and $PGF_{2\alpha}$ that were detected originated from the FBS that was added to the medium. Similar amounts of PGs were present in media supplemented with FBS, but not incubated with cells, whereas media not supplemented by FBS contained no PGs (data not shown).

That HCT-15 cells do not produce PGs was further confirmed by treating these cells with either A23187, arachidonic acid, or mellitin, three known potent stimulators of PG synthesis [29–31]. HCT-15 and HT-29 cells were plated, in the presence of FBS, 24 hr prior to the experiment. Then cells were washed three times with prewarmed PBS and once with medium without FBS. This was followed by incubation for 30 min with medium, containing A23187 (0.01 μg/mL) or arachidonic acid (1 μg/mL) or mellitin (1 μg/mL), without FBS. PGs were then assayed in the culture media. HCT-15 cells did not produce any PGs, whereas, as expected, HT-29 cells responded to stimulation by producing increased amounts of the PGs (3- to 5-fold increases over the baseline) (data not shown).

The functional evidence from the previous experiments regarding the inability of these cells to synthesize PGs was strengthened by the study of the mRNA species corresponding to the two cyclooxygenase enzymes, COX-1 and COX-2. Transcripts from both COX genes were detected by RT-PCR in HT-29 cells but neither was detectable in the HCT-15 cells; samples from both HT-29 and HCT-15 cells were run in parallel with one serving as a positive control for the other (Fig. 1). The transcript for the actin gene was

**TABLE 1. PG levels in the medium when HCT-15 or HT-29 cells were cultured with or without FBS for 24 hr**

| | Prostaglandins (ng/mL) | | | |
|---|---|---|---|---|
| **Cell line** | **$PGE_2$** | **$PGF_{2\alpha}$** | **$PGI_2$** | **$TxA_2$*** |
| HCT-15 | | | | |
| (−) FBS | U† | U | U | U |
| (+) FBS | 0.051 ± 0.012 | 0.030 ± 0.005 | U | ND‡ |
| HT-29 | | | | |
| (−) FBS | 0.051 ± 0.008 | 0.010 ± 0.007 | 0.023 ± 0.011 | U |
| (+) FBS | 0.460 ± 0.011 | 0.078 ± 0.006 | 0.067 ± 0.002 | ND |

Values are means ± SEM from three independent assays.
* $TxA_2$ was assayed as its product, $TxB_2$.
† U = undetectable.
‡ ND = not determined.

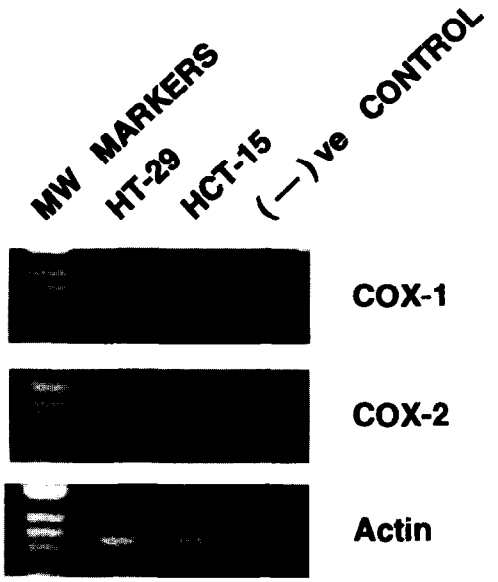


**FIG. 1. Expression of COX-1 and COX-2 in HT-29 and HCT-15 cells. mRNA from HT-29 and HCT-15 cells was reverse-transcribed, and COX-1, COX-2, and β-actin sequences were amplified by PCR generating, respectively, 303, 305, and 318 bp sized fragments. The lane labeled (-)ve control was loaded with the products of a reaction containing no added RNA substrate.**

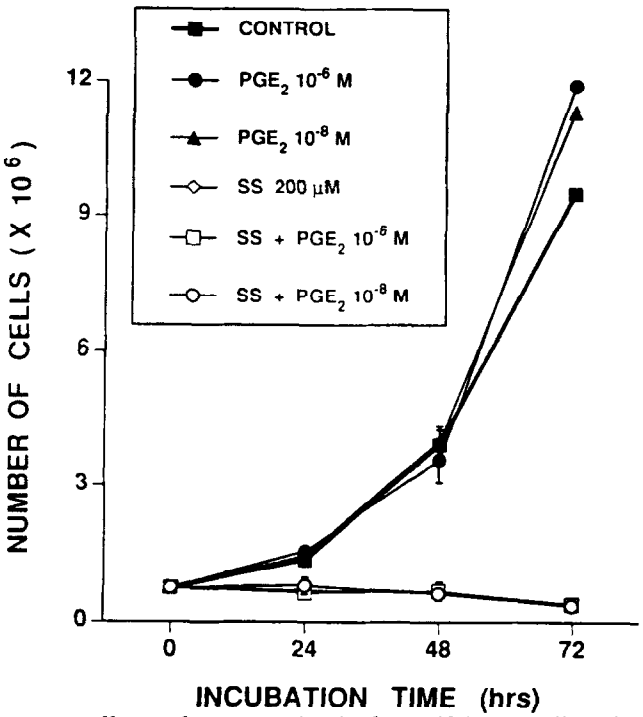


**FIG. 2. Effects of $PGE_2$ and sulindac sulfide on cell proliferation in HCT-15 cells. The cells were cultured in the presence of FBS and counted at 24, 48, and 72 hr. Each value represents the mean ± SEM (N = 4). SS = sulindac sulfide.**

detected in both the cell lines in approximately equal amounts.

### *Effect of PGs on the Antiproliferative Activity of NSAIDs on Colon Cancer Cells*

HCT-15 and HT-29 cells were plated at a density of 0.75 × $10^6$ in P100 tissue culture dishes. Since cells require FBS to be sustained in culture beyond 24 hr, all media were supplemented with 10% FBS, which contains small amounts of PGs. Twenty-four hours after plating, cells were incubated with the NSAID under study and the appropriate PG. Media were changed every day along with addition of fresh compounds. Cells were harvested 24, 48, and 72 hr after the addition of the test compounds.

As expected, sulindac sulfide (200 μM) profoundly reduced the number of HCT-15 cells (Fig. 2); this effect became evident as early as 24 hr after its addition to the culture medium. Concomitant administration of $PGE_2$ at $10^{-6}$ or $10^{-8}$ M did not reverse the effect of sulindac sulfide on the proliferation rate of HCT-15 cells. Treatment of cells with $PGE_2$ for 24 hr prior to adding sulindac sulfide did not prevent the antiproliferative effect of sulindac sulfide. Similarly, $PGF_{2\alpha}$, applied over the same range of concentrations, also failed to reverse the effect of sulindac sulfide on the proliferation rate of HCT-15 cells, when studied for 72 hr (data not shown). Simultaneous addition of $PGE_2$ and $PGF_{2\alpha}$ at a concentration of $10^{-10}$ M also did not reverse the effect of sulindac sulfide on cell proliferation.

Of note, both $PGE_2$ and $PGF_{2\alpha}$ by themselves stimulated the proliferation rate of these cells by 25% over control cells at 72 hr ($P = 0.032$). This finding provides an important control, indicating that both the $PGE_2$ and $PGF_{2\alpha}$ that were added to the culture media were biologically active.

Similar changes were observed in HT-29 cells. Sulindac sulfide (185 μM) suppressed the proliferation rate of HT-29 cells as early as 24 hr after its addition to the medium. Of note, sulindac sulfide at 200 μM had such a profound antiproliferative effect on the HT-29 cells that a lower concentration had to be used. None of the three PGs produced by these cells ($PGE_2$, $PGF_{2\alpha}$, and $PGI_2$) was capable of reversing the antiproliferative effect of sulindac sulfide on HT-29 cells. As was the case with HCT-15 cells, these compounds were added to the media either alone or in various combinations and at the same range of concentrations ($10^{-10}$, and $10^{-8}$, and $10^{-6}$ M). Pretreatment of the cells with $PGE_2$ did not confer any advantage, either. Again, $PGE_2$ and $PGF_{2\alpha}$ stimulated cell proliferation, confirming that they were biologically active.

We also evaluated the effect of PGs on the suppression of the proliferation rate of HCT-15 cells in response to another NSAID, piroxicam, following the same protocol. Treatment of HCT-15 cells with 900 μM piroxicam inhibited their proliferation (81% reduction compared with control at 72 hr). Addition to the culture medium of $PGE_2$ or $PGF_{2\alpha}$ at $10^{-6}$ or $10^{-8}$ M, alone or in combination, failed to reverse the effect of piroxicam (Fig. 3).

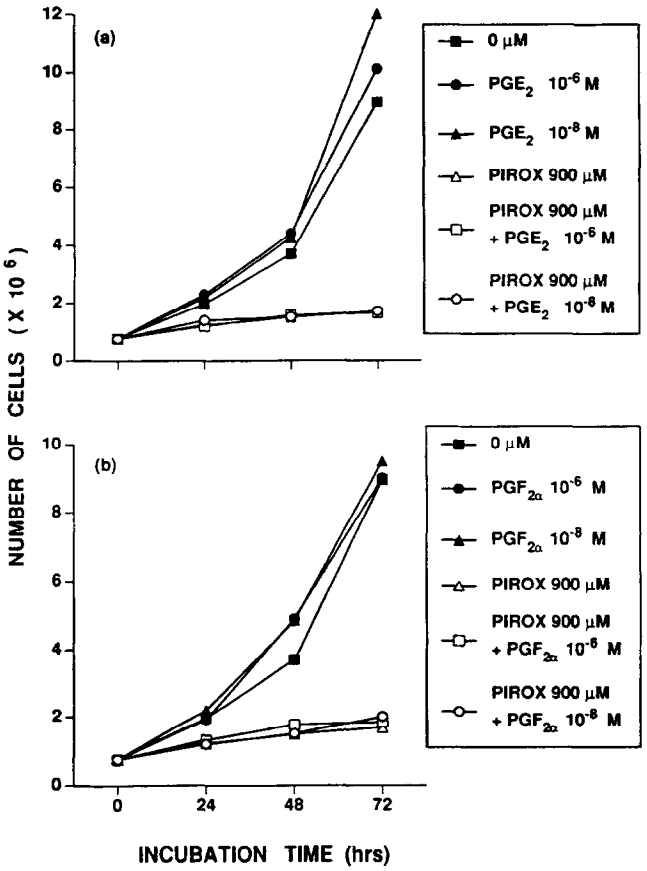


**FIG. 3. Effects of piroxicam, $PGE_2$, and $PGF_{2\alpha}$ on cell proliferation in HCT-15 cells. Cells were cultured and counted at 24, 48, and 72 hr as described in Materials and Methods. Cells were treated with piroxicam (900 μM) with and without the addition of $PGE_2$ (a) or $PGF_{2\alpha}$ (b), both at $10^{-6}$ and $10^{-8}$ M. Each number represents the mean of three separate experiments.**

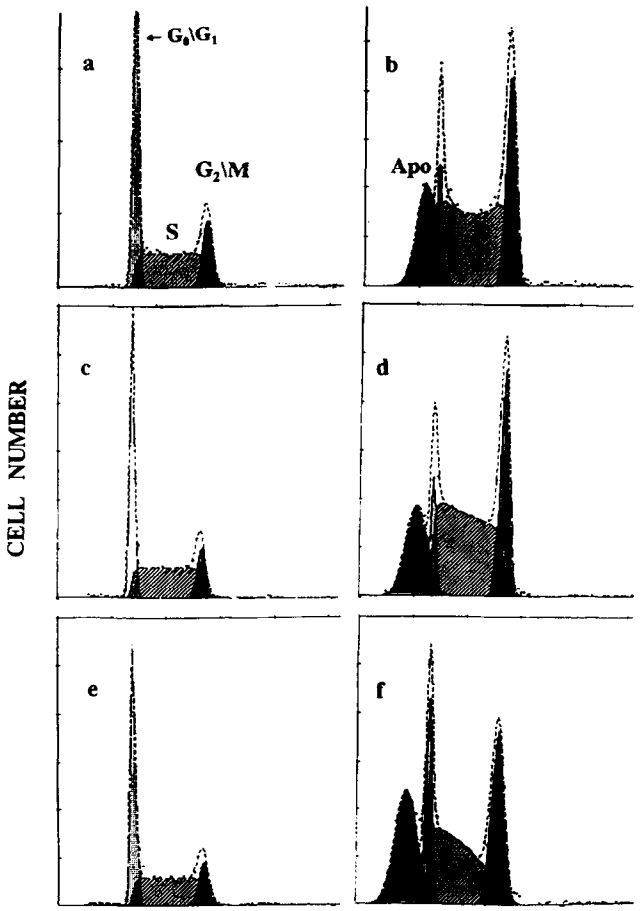


**FIG. 4. DNA histograms of HCT-15 cells treated with PGs and sulindac sulfide. Cells were harvested after 48 hr of treatment with sulindac sulfide and $PGE_2$, as in the text. Key: (a) control; (b) sulindac sulfide, 200 μM; (c) $PGE_2$, $10^{-6}$ M; (d) sulindac sulfide, 200 μM, plus $PGE_2$, $10^{-6}$ M; (e) $PGE_2$, $10^{-8}$ M; and (f) sulindac sulfide, 200 μM, plus $PGE_2$, $10^{-8}$ M. Apo = apoptosis.**

### *Effect of PGs on the Cell Cycle Distribution Changes Induced by NSAIDs*

Aliquots of cell cultures were obtained from the experiments described above, and their distribution in the phases of the cell cycle was determined by flow cytometric analysis.

When HCT-15 cells were grown in the presence of FBS and incubated for up to 72 hr, the NSAIDs induced significant changes in their cell cycle distribution. Sulindac sulfide induced a $G_2$/M arrest within 24 hr of treatment which continued into 48 hr (Fig. 4). This was associated with apoptosis detectable as a subdiploid peak on the DNA frequency histogram (Fig. 4). As is evident from the histograms in Fig. 4, there appears to be an accumulation of cells in the S phase. This probably represents apoptosis of cells in the $G_2$/M and S phases, leading to accumulation of sub-$G_2$/M amounts of DNA, which overlaps with the S phase DNA in the histograms.

When HCT-15 cells were treated with 900 μM piroxicam, an accumulation of cells in the S phase was observed, giving the appearance of an S phase block (data not shown). Addition of $PGE_2$ or $PGF_{2\alpha}$ at a $10^{-6}$ or $10^{-8}$ M concentration simultaneously with either sulindac sulfide or piroxicam failed to prevent these changes. These PGs by themselves did not produce any significant changes in the cell cycle distribution of HCT-15 cells.

Treatment of HT-29 cells with 185 μM sulindac sulfide (following the same protocol as for HCT-15 cells) led to significant changes in their cell cycle distribution. Sulindac sulfide induced a $G_1$ arrest in these cells as opposed to the $G_2$ arrest observed in the HCT-15 cells. After 72 hr of treatment with sulindac sulfide, apoptosis in the form of a subdiploid peak also became evident. The proportion of cells in the $G_0/G_1$ phase increased by 23, 20.5, and 34.5%, at 24, 48, and 72 hr, respectively. The proportion of cells in the S phase remained unchanged while the proportion of cells in the $G_2$/M phase decreased by more than 70% at 72 hr. Addition of either $PGE_2$ or $PGF_{2\alpha}$ at a concentration of $10^{-6}$ or $10^{-8}$ M did not reverse these changes. The PGs by themselves did not induce any significant changes in the

cell cycle distribution of HT-29 cells compared with controls.

### *Failure of PGs to Reverse NSAIDs-Induced Apoptosis in Colon Cancer Cell Lines*

NSAIDs induce apoptosis in the HT-29 colon cancer cell line [17, 18]. We observed a similar phenomenon in HCT-15 cells, and we examined whether this effect was dependent on the inhibition of PG synthesis by NSAIDs.

Apoptosis was evaluated in parallel experiments by (a) evaluating the cellular morphology after staining the cells with acridine orange, (b) assessing DNA degradation by flow cytometric analysis, and (c) examining the degradation of genomic DNA by agarose gel electrophoresis.

When these two cell lines were studied for up to 72 hr, sulindac sulfide at 200 μM induced apoptosis in both HCT-15 and HT-29 cells. As summarized in Table 2, the apoptosis induced by sulindac sulfide in HCT-15 cells was 13% at 24 hr, 42% at 48 hr and 44% at 72 hr as assessed by acridine orange staining. Figure 5 shows the morphologic changes of apoptosis in these cells. In HT-29 cells, the corresponding values were 5% at 24 hr, 7% at 48 hr, and 23% at 72 hr. Flow cytometric analysis indicated similar changes (Table 2 and Fig. 4). The addition of $PGE_2$ or $PGF_{2\alpha}$ at $10^{-6}$, $10^{-8}$, or $10^{-10}$ M simultaneously with sulindac sulfide did not reverse this effect. When PGs were added alone to the cells, there was not effect on apoptosis as assessed by either method.

**TABLE 2. Effect of $PGE_2$ on apoptosis in HCT-15 and HT-29 cells induced by sulindac sulfide**

| | Flow cytometry* | | | Acridine orange† | | |
|---|---|---|---|---|---|---|
| | 24 hr | 48 hr | 72 hr | 24 hr | 48 hr | 72 hr |
| HCT-15 | | | | | | |
| Control | U‡ | U | U | 2 | 1 | 1 |
| SS§ | 7 | 33 | 27 | 13 | 42 | 44 |
| SS + $PGE_2$ $10^{-6}$ M | 12 | 34 | 43 | 14 | 52 | 55 |
| SS + $PGE_2$ $10^{-8}$ M | 8 | 27 | 29 | 10 | 51 | 54 |
| $PGE_2$ $10^{-6}$ M | U | U | U | 3 | 2 | 3 |
| $PGE_2$ $10^{-8}$ M | U | U | U | 3 | 0.5 | U |
| HT-29 | | | | | | |
| Control | U | U | U | U | U | U |
| SS | U | U | 45 | 5 | 7 | 23 |
| SS + $PGE_2$ $10^{-6}$ M | U | U | 38 | 5 | 6 | 20 |
| SS + $PGE_2$, $10^{-8}$ M | U | U | 42 | 4 | 7 | 25 |
| $PGE_2$ $10^{-6}$ M | U | U | | U | U | U |
| $PGE_2$ $10^{-8}$ M | U | U | | U | U | U |

Apoptosis was determined by flow cytometry and acridine orange staining as described in Materials and Methods.

* Percent of cells exhibiting a subdiploid peak of DNA when analyzed by flow cytometry.

† Percent of cells exhibiting morphologic changes characteristic of apoptosis when stained with acridine orange.

‡ U = undetectable.

§ SS = sulindac sulfide.

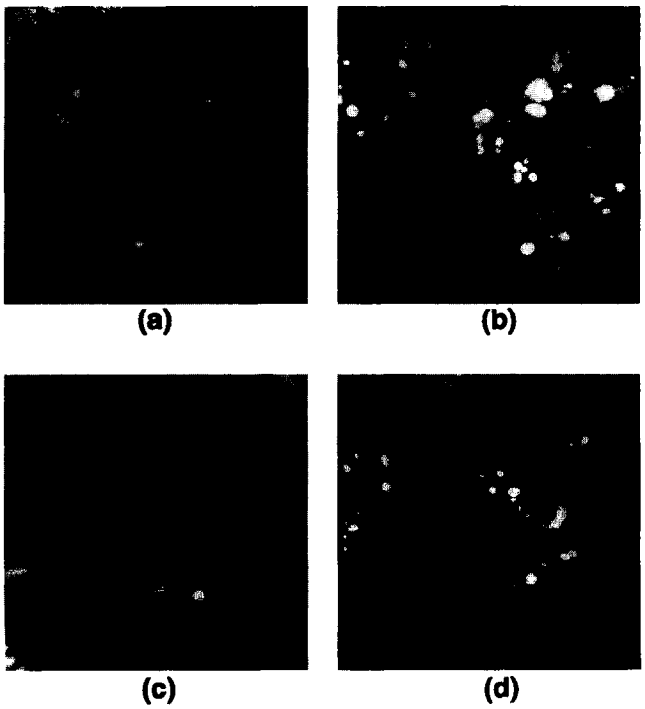


**FIG. 5. HCT-15 cells evaluated for apoptosis by acridine orange staining. Cells were cultured in the presence of FBS and harvested at 48 hr as described in Materials and Methods. Key: (a) control cells; (b) cells treated with sulindac sulfide, 200 μM; (c) cells treated with $PGE_2$, $10^{-6}$ M; and (d) cells treated with both $PGE_2$, $10^{-6}$ M, and sulindac sulfide, 200 μM. Photomicrographs (b) and (d) show morphological changes typical of apoptosis: cytoplasmic and nuclear shrinkage, chromatin condensation, and apoptotic bodies. (40× Magnification.)**

Finally, the degradation of genomic DNA, a hallmark of apoptosis, was also analyzed in response to sulindac sulfide treatment by size fractioning the genomic DNA of HCT-15 cells. As seen in Fig. 6, sulindac sulfide at 200 μM degraded the DNA of these cells, resulting in a prominent ladder below the predominant genomic DNA band. Once more, the presence of $PGE_2$ or $PGF_{2\alpha}$ did not prevent these changes.

## DISCUSSION

Our findings demonstrate that the NSAIDs sulindac sulfide and piroxicam inhibited the proliferation rate of two colon adenocarcinoma cell lines by a mechanism independent of their ability to inhibit PG synthesis. Two lines of evidence supported this conclusion: (a) the same effect occurred in cells that produce PGs as in cells that do not, and (b) exogenously added PGs did not reverse the effect of NSAIDs.

The two cell lines, HT-29 and HCT-15, differed in their ability to produce PGs; the former produced PGs but the latter did not. When HCT-15 cells were cultured in the

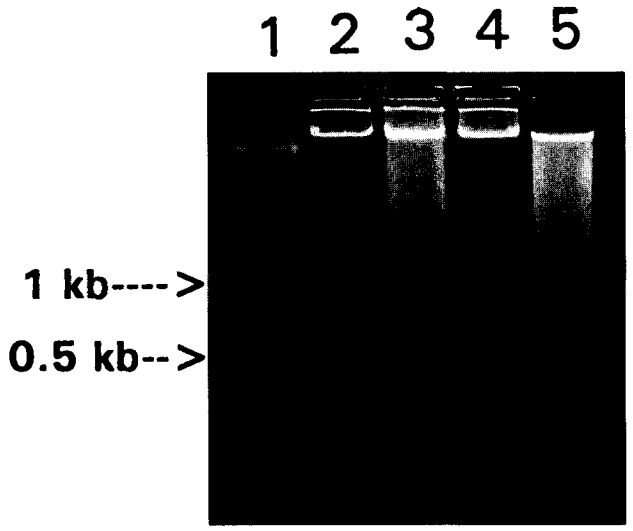


**FIG. 6. Electrophoresis of genomic DNA from HCT-15 cells treated with sulindac sulfide, $PGE_2$, and $PGF_{2\alpha}$. Genomic DNA was isolated from HCT-15 cells and fractionated on 1.8% agarose gels, as described in Materials and Methods. HCT-15 cells treated with sulindac sulfide (200 μM) revealed the characteristic ladder pattern, a hallmark of apoptosis, which was not prevented by simultaneous addition of $PGE_2$ and $PGF_{2\alpha}$ at $10^{-10}$ M. Lane 1 = molecular weight marker; lane 2 = control; lane 3 = sulindac sulfide, 200 μM; lane 4 = $PGE_2$ + $PGF_{2\alpha}$; and lane 5 = sulindac sulfide plus $PGE_2$ and $PGF_{2\alpha}$.**

absence of serum, their media contained no detectable PGs. This is in contrast to HT-29 cells which constitutively synthesized three PGs. These findings are consistent with previous reports on the biosynthetic profile of these cell lines with respect to PGs [32]. Our conclusion was strengthened further by two observations: (a) three different potent stimulators of PG synthesis failed to induce PG production by the HCT-15 cells, while stimulating PG production by the HT-29 cells, and (b) transcripts of the two known cyclooxygenase genes, COX-1 and COX-2, were not detectable in HCT-15 cells, while they were present in HT-29 cells.

These two cell lines, despite the differences in their ability to produce PGs, responded in a similar fashion to NSAIDs with respect to cell proliferation. Therefore, this suggests that inhibition of PG synthesis by NSAIDs is not critical for their inhibitory effect on cell proliferation.

The failure of exogenously added PGs to reverse the effect of NSAIDs further supports this conclusion. In these experiments, PG concentrations ranged from those which would merely replace the endogenous production if it were inhibited completely by the NSAIDs to concentrations $10^4$ times higher. Pretreatment of the cells with PGs or the combined administration of the two compounds also failed to influence the effects of the NSAIDs. As noted earlier, both $PGE_2$ and $PGF_{2\alpha}$ stimulated the proliferation of both HCT-15 and HT-29 cells. This effect was seen consistently in all experiments. In view of the unstable nature of these compounds, this indicates the presence of active interaction between the cells and these compounds, thus providing a reassuring positive control in these experiments.

We also examined two parameters that contribute to the proliferation rate of these cells in culture; the distribution of these cells in the cell cycle and apoptosis. The NSAIDs profoundly affected both of these parameters. These effects also appeared to be independent of their effects on PG synthesis.

Both HCT-15 and HT-29 cells changed their cell cycle distribution in response to sulindac sulfide and piroxicam, albeit these changes were not identical between the two cell lines. When HCT-15 cells were treated with sulindac sulfide, their cell cycle arrested in $G_2$/M phase, while similar treatment of HT-29 cells induced a $G_1$ arrest. This was not surprising considering that these are two different cell lines. Nevertheless, in either case the exogenous administration of PGs had no effect on the distribution of cells in the cell cycle in response to treatment with either sulindac sulfide or piroxicam.

Sulindac sulfide induced apoptosis, and this probably contributed to the overall reduction in the proliferation rate of these cells. As with the cell cycle changes, (a) apoptosis was observed in both the HT-29 and HCT-15 cells, the latter of which do not produce PGs, and (b) PGs were unable to overcome the effect of NSAIDs on apoptosis. These two findings indicate that this effect, too, is independent of the ability of these NSAIDs to inhibit PG synthesis.

The NSAIDs used in this study belong to different chemical classes: sulindac sulfide is a member of the "acetic acid group," and piroxicam of the "enolic acid group." These findings, therefore, argue against an effect restricted to a single compound, and raise the possibility that our conclusion may apply to many, if not all, NSAIDs.

Our finding that NSAIDs can affect the proliferation rate of colon adenocarcinoma cells *in vitro* as well as two contributing parameters, cell cycle distribution and apoptosis, by a mechanism other than inhibition of PG synthesis, is not totally unexpected. Over the last several years, evidence has been accumulating that the mechanism of some of the actions of NSAIDs is not related to their ability to inhibit PG synthesis. For example, the doses of NSAIDs required to inhibit the cyclooxygenase are much lower than those required for their anti-inflammatory effect [13]. In addition, the NSAID sodium salicylate does not inhibit cyclooxygenase at the concentrations used clinically, but it still is an effective anti-inflammatory agent. In addition, sodium salicylate and aspirin inhibit, via a PG-independent mechanism, the activation of NF-kB, an inducible eukaryotic transcription factor of the Rel family [33], which therefore inactivates certain genes involved in the immune and inflammatory response [34]. Finally, two observations have been made that directly relate to the present study: (a) PGs do not prevent growth inhibition by NSAIDs in human fibroblasts and rat hepatoma cells *in vitro* [35], and (b) $PGE_2$ given to rats concomitantly with indomethacin does not

reverse the tumor-reducing effect of indomethacin in these animals [36]. These observations support our result that indicates that several effects of NSAIDs, including those that we are studying, are PG independent.

It is of interest that the concentrations of sulindac sulfide used in our experiments, can, in theory, be achieved in the colon of humans. Humans given sulindac, at doses that regress polyps in FAP patients, achieve plasma concentrations of sulindac sulfide of about 10–15 μM [37, 38]. A large fraction of sulindac is converted to sulindac sulfide, its active metabolite, in the colon by colonic bacteria [39]. This results in a high lumenal concentration of sulindac sulfide in the colon [40]. In addition, animal studies have shown that sulindac sulfide is concentrated in the mucosa of the colon at levels several-fold higher than those in the serum [41]. Colonic epithelial cells could thus be exposed to concentrations up to 20-fold higher than those in serum [40]. Since the tissue to plasma level ratio of sulindac sulfide in the colon is in the range of 10–20 [40, 41], it is conceivable that the concentrations used in our study could be achieved in the human colon.

In summary, our findings strongly, if not conclusively, indicate that the effect of some NSAIDs on the proliferation of colon cells in culture, and on the two contributing parameters of cell proliferation, i.e., cell cycle and apoptosis, is exerted independent of their inhibitory effect on PG synthesis. A mechanism(s) unrelated to inhibition of PG synthesis that affects the cell cycle phase distribution and apoptosis in response to NSAIDs is currently being sought.

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