A Novel Mechanism for Aspirin-mediated Growth Inhibition of Human Colon Cancer Cells

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ABSTRACT

Purpose: The molecular mechanisms by which aspirin and other nonsteroidal anti-inflammatory drugs exert chemopreventive effects in colon cancer are unclear and complex. Current investigations focus on the chemopreventive properties of nonsteroidal anti-inflammatory drugs, independent of their ability to inhibit cyclooxygenase (COX) activity, and presumably, identification of non-COX pathways will suggest new targets for clinical use. It was demonstrated recently that aspirin results in reduced microsatellite instability in colorectal cancer cells. We hypothesized that aspirin treatment might alter expression of DNA mismatch repair (MMR) proteins, representing another potential non-COX mechanism for its action.

Experimental Design: In this study, we have examined the effects of aspirin on the cellular growth rates, MMR protein levels, cell cycle analysis and apoptosis in MMR-deficient (HCT116) and MMR-proficient (HCT116+chr3 and SW480) human colon cancer cell lines.

Results: We found that treatment with aspirin inhibited the growth of these three cancer cell lines. In HCT116+chr3 cells, treatment with 1 mM of aspirin increased expression of the hMLH1 and hPMS2 proteins by 2.5-fold and 2-fold, respectively, and increased expression of the hMSH2 and hMSH6 proteins by 2–3-fold. For SW480 cells, treatment with 1 and 5 mM of aspirin increased expression of the hMLH1 and hPMS2 proteins by 2–4-fold and 3–5-fold, respectively, and increased expression of the hMSH2 and hMSH6 proteins by 3–7-fold. For all three of the cell lines, treatment with 1 and 2.5 mM of aspirin induced apoptosis at 48 and 72 h. Aspirin induced G0/G1 cell cycle arrest in HCT116 cells.

Conclusions: We conclude that aspirin acts through COX-independent mechanisms by resulting in an increase in MMR protein expression and subsequent apoptosis, which might serve as an additional means of growth inhibition in aspirin-treated human colon cancer cells.

INTRODUCTION

The shortcomings of current treatment modalities for common epithelial cancers have revived interest in the area of cancer prevention. This is particularly true for colon cancer with the potential chemopreventive activities of NSAIDs (1). Increasing evidence from human epidemiological studies, animal models, and in vitro experiments reveal that the administration of NSAIDs represents a viable option for the chemoprevention of colon cancer. These data indicate that use of NSAIDs, including aspirin, is inversely associated with the risk of colorectal cancer, and clinical trials among patients with familial adenomatous polyposis show that use of NSAIDs can lead to the regression of colorectal adenomas (2). In addition, data derived from various animal models of chemical carcinogenesis also suggest that NSAIDs exert protective effects in the stomach and esophagus.

The molecular mechanisms underlying the chemopreventive effects of NSAIDs are less well understood and are a matter of ongoing debate. Protection might occur through several pathways, including cell cycle arrest, induction of apoptosis, and inhibition of angiogenesis. One most widely accepted mechanism for the anticancer effect of NSAIDs is the reduced PG synthesis by inhibiting COX activity. On the contrary, not all of the NSAIDs reduce PG synthesis equivalently (3, 4), and, often, much higher doses are required to produce anti-inflammatory effects than are required to inhibit PG synthesis. NSAIDs have growth inhibitory effects against colon cancer cell lines that do not express COX-1 or COX-2 enzymes (5), and against mouse embryo fibroblasts that are null for both COX-1 and COX-2 genes (6). Such observations are inconsistent with the conventional belief that NSAIDs act primarily or exclusively by inhibiting PG synthesis.

NSAIDs mediate their antineoplastic properties by affecting mechanisms other than inhibition of COX, as NSAIDs interact with a variety of intracellular processes (5, 7, 8). By binding to inhibitor of nuclear factor-κB kinase β, aspirin inhibits the activation, and therefore, the DNA binding activity of nuclear factor-κB (9, 10). Aspirin induces apoptosis by activating p38 kinase in normal human fibroblasts (11). Several

1 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; PG, prostaglandin; COX, cyclooxygenase; MMR, mismatch repair; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; 7-AAD, 7-amino-actinomycin-D.
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NSAIDs are known to be activators of the nuclear receptor peroxisome proliferator-activated receptor γ, demonstrating the potential that NSAIDs directly affect transcription (12). Another possible target for the activity of NSAIDs is suggested by the reduced microsatellite instability seen in aspirin- or sulin-dac-treated colorectal cancer cells that lack DNA MMR activity (13).

Such observations emphasize the importance of identifying COX-independent biochemical pathways for aspirin and related NSAIDs, as knowledge of these pathways should reveal promising new targets for designing antineoplastic or chemopreventive agents. In this study, we investigated the effect of aspirin on the expression of DNA MMR proteins in human colon cancer cell lines that are deficient or proficient in a subset of these genes. We demonstrate that aspirin treatment increases expression of the hMLH1, hMSH2, hMSH6, and hPMS2 proteins in DNA MMR-proficient colon cancer cell lines. These results suggest that regulation of the MMR system might be another COX-independent mechanism by which aspirin acts to prevent the development of colon cancer.

MATERIALS AND METHODS

Cell Lines and Cell Culture. The human colon cancer cell lines HCT116 (DNA MMR-deficient) and SW480 (MMR-proficient) were obtained from the American Type Culture Collection. HCT116 contains homozygous mutations in hMLH1 resulting in a truncated, nonfunctional protein (14). A derivative line, HCT116+chr3, was made MMR-proficient by the stable transfer of chromosome 3 bearing a wild-type copy of the hMLH1 gene (15) and was grown in the presence of 400 μg/ml G418 in the culture medium. All of the cell lines were cultured in Iscove’s modified Dulbecco’s medium with 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY) and maintained at 37°C in 5% CO2.

Aspirin Treatment. We dissolved aspirin (Sigma, St. Louis, MO) in 1 m Tris-HCl (pH 7.5) to a stock concentration of 1 m and adjusted the pH to 7.2 with 4 m HCl. Twenty-four h before aspirin treatment, exponentially growing human colon cancer cell lines were seeded at a density of 105 cells/100-mm culture dish (Becton Dickinson, Franklin Lakes, NJ). The cells were washed once with PBS. All of the cell lines were treated for 24, 48, and 72 h by adding various volumes of stock to obtain final concentrations of 1, 2.5, 5, or 10 mM of aspirin. Control cells were treated with an equivalent volume of Tris-HCl.

Estimation of Growth Rates. The proliferation rates of cells treated with aspirin were determined by comparing cell numbers of aspirin-treated and untreated control cells. All three of the cell lines were plated at a density of 0.5 × 105 cells/100-cm tissue culture dish. At 24 h after seeding, cells were treated with different concentrations of aspirin for 24, 48, or 72 h. At the end of each treatment interval, the cells were trypsinized, washed, and resuspended in 1 ml of PBS. Aliquots of cells were counted with a hemocytometer and tested for viability by trypan blue dye exclusion.

MTT Assay. The effect of aspirin on cellular viability was evaluated by an assay that is based on the activity of mitochondrial dehydrogenase, which cleaves the yellow dye MTT to form purple formazan crystals, a conversion that occurs only in living cells. Human colon cancer cell lines (HCT116, HCT116+chr3, and SW480) were plated in 96-well microtiter plates at a density of 104 cells/well in a final volume of 100 μl of Iscove’s modified Dulbecco’s medium. Twenty-four h after the initial seeding, cells were treated with aspirin. Untreated cells (appropriate volumes of buffer solution added) served as controls. After treatment, the cells were incubated for 3 h at 37°C with a solution of MTT (Sigma) at a concentration of 50 μg/100 μl. The cells were lysed for 12 h at room temperature in a buffer containing 10% SDS and 0.01 M HCl (16). For each sample, the absorbance of the reduced intracellular formazan product was read at 570 nm in a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA). Each assay was performed in triplicate and repeated three times.

Western Blot Analysis of MMR Proteins. Cells were treated for 30 min in lysis buffer (17), sonicated for 1–2 min on ice, and centrifuged at 10,000 × g for 10 min to obtain clear supernatants. The protein concentration of whole cell lysates was measured with the BCA protein assay kit (Pierce, Rockford, IL) following the manufacturer’s instructions. Protein aliquots (50 μg) were then mixed with an identical amount of Laemmli gel loading buffer and placed in a boiling water bath for 5 min. Proteins were separated by 8% SDS-PAGE and transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked for 1 h with 5% nonfat milk in PBS-Tween 20. Subsequently, the membranes were probed with anti-hMLH1, hMSH2, hMSH6, and hPMS2 antibodies as described in detail previously (18). Blots were washed with PBS-Tween 20 and incubated with horseradish peroxidase-conjugated secondary antibody or IgG antibodies. The membranes were developed with the ECL Plus chemiluminescence system (Amersham, Arlington Heights, IL). We ensured that samples contained equal amounts of protein by preparing SDS-PAGE gels in duplicate and staining one of each set with Coomassie blue. We used a sensitive spot-densitometry imager (Alpha Innotech Corporation, San Leandro, CA) to calculate changes in protein expression by normalizing the individual band intensities of treated samples with the respective untreated controls.

Semiquantitative Multiplex Reverse Transcription-PCR for MMR Genes. Each cell line was plated at a density of 105 cells/10-cm dish and treated with or without aspirin 24 h after seeding. Total cellular RNA was extracted using TRIzol (Life Technologies, Inc.) according to the manufacturer’s instructions. cDNA was reverse transcribed from 1 μg of total cellular RNA in 20-μl reactions containing 25 μg/ml of a random hexamer (Roche Molecular Biochemicals, Indianapolis, IN), 10,000 units/ml of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc.), 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 0.4 mM deoxy nucleoside triphosphates, 2,000 units/ml of RNase inhibitor (Stratagene, La Jolla, CA), and 8.5 μl of diethyl pyrocarbonate-treated water. Subsequently, we determined the mRNA expression of six DNA MMR genes (hMLH1, hMSH2, hPMS1, hPMS2, hMSH6, and hMSH3) by multiplex-PCR as described by us previously (18).

Apoptosis Determination. Each cell line was plated at a density of 106 cells/10-cm dish and treated with 1 or 2.5 mM of
Aspirin Increases the Steady-State Level of MMR Proteins in Colon Cancer Cells. In all of the cell lines, the steady-state levels of the MMR proteins hMLH1, hPMS2, hMSH2, and hMSH6 were determined after aspirin treatment. Aspirin treatment did not significantly change the expression of any of these four MMR proteins at the 24- or 48-h treatment interval in any cell line. As expected, MMR-deficient HCT116 showed no detectable expression of hMLH1 or hPMS2, as reported previously (18). MMR-proficient HCT116+chr3 cells, which express the hMLH1 protein by complementation of a wild-type gene on chromosome 3, expressed the hPMS2 protein. HCT116+chr3 cells that were treated with 1 mM of aspirin showed a significant, 2.5-fold increase in expression of the hMLH1 protein (Fig. 3). A similar increase in hMLH1 protein expression was observed in SW480 cells at 1 and 2.5 mM (4-fold) and 5 mM (2.5-fold) aspirin concentrations. HCT116+chr3 cells showed a 2-fold increase in expression of the hPMS2 protein with 1 mM of aspirin. The increased expression of the hPMS2 protein in SW480 cells was in similar

RESULTS

Aspirin Inhibits the Growth of Human Colon Cancer Cell Lines Irrespective of MMR Status. Aspirin treatment induced a profound concentration- and time-dependent reduc-

tion in the proliferation rate of each of the three colon cancer cell lines, as indicated by the viable cell counts after each treatment regimen. However, 72 h after treatment, inhibition of the proliferation rate of HCT116+chr3 cells was significantly less than inhibition of HCT116 cells at 1.0 mM (14.3% vs. 40.68%; P < 0.05) and 2.5 mM (27.2% vs. 47.93%; P < 0.05) of aspirin. After 24 and 48 h of aspirin treatment, statistically nonsignificant differences were observed in the growth rates of these two cell lines. The growth curves for DNA MMR-proficient SW480 cells were identical to the growth curves observed for HCT116+chr3 cells (Fig. 1).

The MTT assay also demonstrated growth inhibition in all of the cell lines, and these results were concordant with the reduced proliferation rates after aspirin treatment (Fig. 2). After 24 h of aspirin treatment, all of the cell lines, irrespective of their DNA MMR status, showed a 20–30% reduction in cell viability. This reduction in cell viability increased after 48 and 72 h of aspirin treatment. When treated with up to 7.5 mM aspirin, MMR-proficient (HCT116+chr3 and SW480) and MMR-deficient (HCT116) cell lines showed no significant differences in cell viability. Although, after 48 and 72 h of treatment with 10 mM aspirin, the survival of HCT116+chr3 and SW480 cells was significantly better when compared with the survival of HCT116 cells (P < 0.03).

Aspirin Increases the Steady-State Level of MMR Proteins in Colon Cancer Cells. In all of the cell lines, the steady-state levels of the MMR proteins hMLH1, hPMS2, hMSH2, and hMSH6 were determined after aspirin treatment. Aspirin treatment did not significantly change the expression of any of these four MMR proteins at the 24- or 48-h treatment interval in any cell line. As expected, MMR-deficient HCT116 showed no detectable expression of hMLH1 or hPMS2, as reported previously (18). MMR-proficient HCT116+chr3 cells, which express the hMLH1 protein by complementation of a wild-type gene on chromosome 3, expressed the hPMS2 protein. HCT116+chr3 cells that were treated with 1 mM of aspirin showed a significant, 2.5-fold increase in expression of the hMLH1 protein (Fig. 3). A similar increase in hMLH1 protein expression was observed in SW480 cells at 1 and 2.5 mM (4-fold) and 5 mM (2.5-fold) aspirin concentrations. HCT116+chr3 cells showed a 2-fold increase in expression of the hPMS2 protein with 1 mM of aspirin. The increased expression of the hPMS2 protein in SW480 cells was in similar

aspirin. After treatment, cells were harvested by trypsinization and washed once with 10 ml of PBS. We used the Annexin V-Cy5 apoptosis detection kit (PharMingen, San Diego, CA) according to the manufacturer’s instructions to stain the apoptosis marker, cell surface phosphatidylserine. Briefly, cells were resuspended in 200 μl of 1× Annexin binding buffer [10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl2 (pH 7.4)] and stained with 5 μl of Annexin V-Cy5 (1 μg/ml). After a 15-min incubation at room temperature, the cells were incubated with 10 μl of 7-AAD for another 15 min at 25°C in the dark. The cell suspensions were gently centrifuged, and 400 μl of 1× Annexin binding buffer was added. The cells were analyzed by flow cytometry within 1 h of 7-AAD staining. Appropriate controls were used to subtract background counts. We used the FACScalibur Flow Cytometer (Becton Dickinson, San Jose, CA) for two-color analysis of apoptosis. Fluorescence compensation was adjusted to minimize overlap of the Cy5 and 7-AAD signals.

Cell Cycle Analysis. The effect of aspirin treatment on cell proliferation was evaluated by measuring the distribution of cells in different phases of the cell cycle by flow cytometry. This determination was based on the measurement of the DNA content of nuclei labeled with propidium iodide (19). Cell suspensions from either aspirin-treated or untreated cell cultures were prepared by trypsinizing the cells and washing them twice with cold PBS. Cells were resuspended at a density of 10⁶ cells/ml in cold PBS and fixed with 75% ethanol overnight at −20°C. Ethanol-fixed cells were washed with cold PBS, resuspended in 300 μl of PBS containing 0.15% boiled and renatured RNase A (Sigma) at 37°C for 30 min, and stained with 80 μg/ml of propidium iodide for 30 min. Cells were analyzed for DNA content with the FACScalibur Flow Cytometer (Becton Dickinson) at an excitation of 488 nm with detection at 620 nm for red fluorescence. Cell cycle data were analyzed with the Multicycle Software Autofit Version 2.50 (Phoenix Flow Systems, San Diego, CA).

Statistical Analyses. Statistical analyses were performed using the Student t test. For each test, P < 0.05 were considered statistically significant.
proportion as the increase observed for the hMLH1 protein with 1 and 5 mM of aspirin.

On aspirin treatment, HCT116 cells did not show changed expression of the hMSH2 and hMSH6 proteins. However, when treated with 1 mM aspirin, HCT116+chr3 cells showed 2–3-fold increased expression of both the hMSh2 and hMSH6 proteins when compared with the band intensities of untreated control cells (Fig. 4). On the other hand, SW480 cells showed substantially increased expression of the hMSh2 protein at 1 mM (6.5-fold), 2.5 mM (8-fold), and 5 mM (4-fold) aspirin concentrations. Parallel increases in the expression level of the hMSH6 protein (6–7.5-fold) were observed, but the magnitude of this increase was little less than the change in hMSH2 expression that was seen in SW480 cells.

To evaluate whether aspirin affects transcriptional regulation of the MMR system, we used quantitative multiplex reverse transcription-PCR to determine the mRNA expression of key MMR genes. RNA was reverse transcribed to obtain cDNA, which was amplified by PCR with primers for six MMR genes and for β-actin, as a control gene (18). Aspirin, at all of the concentrations used, did not significantly alter the expression of any of the MMR genes in these colon cancer cell lines (data not shown).

**Aspirin Induces More Profound Apoptosis in MMR-proficient Cells Than in MMR-deficient Cells.** To additionally investigate the growth-inhibitory mechanism of aspirin in DNA MMR-deficient and -proficient human colon cancer cells, and to determine whether the observed increase in the expression of MMR proteins was attributable to the apoptotic machinery, we stained cells with Annexin V-Cy5 to detect cell surface phosphatidylserine, a marker for early apoptosis in cultured cells. Two-color staining with Annexin V-Cy5 and 7-AAD was used to differentiate the fraction of cells undergoing early ap-
optosis and subsequent death (Fig. 5). We observed a dose-dependent increase in apoptosis for all three of the cell lines treated with 1 and 2.5 mM of aspirin. After 48 h of treatment, this increase was greater in DNA MMR-proficient HCT116 and SW480 cells than in MMR-deficient HCT116 cells (Fig. 6). At both concentrations, aspirin increased the total cell death as shown by positive double staining with Annexin-Cy5 and 7-AAD. Interestingly, in HCT116+chr3 and SW480 cells ($P < 0.04$) than in MMR-deficient HCT116 cells (Fig. 6). At both concentrations, aspirin increased the total cell death as shown by positive double staining with Annexin-Cy5 and 7-AAD. Interestingly, in
Aspirin Increases MMR Protein in Colon Cancer Cells

Table 1. Effect of aspirin treatment on distribution of cells in various cell cycle phases. Values represent mean percentages (±SD) in various cell lines after treatment with aspirin.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G_0/G1</td>
<td>S</td>
</tr>
<tr>
<td>HCT116</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM</td>
<td>46.88 ± 5.77</td>
<td>34.77 ± 4.15</td>
</tr>
<tr>
<td>51.88 ± 0.028</td>
<td>30.43 ± 1.09</td>
<td>17.7 ± 1.13</td>
</tr>
<tr>
<td>1 mM</td>
<td>54.23 ± 3.10 *</td>
<td>29.45 ± 4.17</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>64.69 ± 6.61 *</td>
<td>24.46 ± 9.53</td>
</tr>
<tr>
<td>HCT116 + chr3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM</td>
<td>56.41 ± 0.417</td>
<td>31.24 ± 1.74</td>
</tr>
<tr>
<td>1 mM</td>
<td>58.12 ± 0.113</td>
<td>30.46 ± 1.20</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>63.49 ± 1.57</td>
<td>26.06 ± 2.63</td>
</tr>
<tr>
<td>SW480</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 mM</td>
<td>46.73 ± 1.31</td>
<td>38.38 ± 2.15</td>
</tr>
<tr>
<td>1 mM</td>
<td>46.45 ± 0.07</td>
<td>39.37 ± 0.33</td>
</tr>
<tr>
<td>2.5 mM</td>
<td>46.19 ± 1.54</td>
<td>40.97 ± 2.02</td>
</tr>
</tbody>
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*P < 0.05 when the values were compared with the untreated control cell lines.

p < 0.01 when the values were compared with the untreated control cell lines.

HCT116-chr3 and SW480 cells, after 72 h of aspirin treatment, there was either no increase or a small decline in the number of cells undergoing apoptosis suggesting that apoptosis is an early event. This interpretation was corroborated by the increase in cells that were positive for both Annexin V-Cy5 and 7-AAD. For MMR-deficient HCT116 cells, after 72 h of treatment with 1 and 2.5 mM of aspirin, the apoptotic population did not significantly increase, which indicates that apoptosis is probably a late event and is preceded by other mechanisms of growth inhibition.

Aspirin Arrests Human Colon Cancer Cells in G_0/G_1 Cell Cycle Phase. To study the effect of aspirin treatment on cell populations in different phases of the cell cycle, we treated exponentially growing cells with 1 or 2.5 mM of aspirin for 48 and 72 h, as the maximal growth inhibitory effects were observed at these time points. Using conventional DNA flow cytometry, we determined the effect of aspirin on the distribution of cells in various phases of the cell cycle. In untreated HCT116 and HCT116+chr3 cells, 55–60% of cells were in the G_0/G_1 phase, 25–30% were in S phase, and the remaining cells were in the G_2/M phase (Table 1). However, in HCT116 cells, after 48 h, the presence of 1 or 2.5 mM of aspirin resulted in an accumulation of cells in G_0/G_1, and arrest continued at 72 h. These results were dose-dependent and were paralleled by a decrease in the percentage of S and G_2/M phase cells. SW480 and HCT116+chr3 cells, both of which are MMR-proficient, did not show cell arrest at any aspirin concentration or time point.

DISCUSSION

The results of this study shed additional insight into the complex mechanisms of aspirin action in colon carcinogenesis. We show that aspirin inhibits the growth of all human colon cancer cell lines; however, DNA MMR-proficient cells are more resistant to the effects of aspirin when compared with MMR-deficient cells. We also observed that treatment with 1.0 mM aspirin in the MMR-proficient HCT116+chr3 and SW480 cell lines increased the expression of most MMR proteins, and this increase was more pronounced in SW480 cells. We additionally provide evidence that aspirin-induced inhibition of the growth of MMR-proficient and -deficient cells is achieved through different growth regulatory mechanisms.

Aspirin and related NSAIDs have attracted considerable attention as compounds that might be of potential benefit in the chemoprevention of cancer (20, 21). However, there is a considerable debate regarding the molecular mechanisms by which aspirin and related NSAIDs inhibit tumor formation and/or growth. One potential mechanism of NSAID action involves inhibition of COX activity, which limits tumorogenesis by reducing the production of mutagens that result from arachidonic acid metabolism (22), by reducing the synthesis of immunosuppressive PGs (23) or by both mechanisms.

NSAIDs have numerous targets other than COX by which they might inhibit tumor growth. For example, inhibition of glycolysis, uncoupling of oxidative phosphorylation, inhibition of nuclear factor κB, disruption of a number of signaling pathways, as well as the inhibition of a many other enzymes, including cyclic AMP-dependent protein kinase, have been attributed to NSAIDs (9, 24, 25).

To date, no study has reported whether aspirin or related NSAIDs are responsible for differential inhibition of growth rates on the basis of DNA MMR proficiency. Here, we demonstrate that although aspirin inhibits the growth of both MMR-deficient (HCT116) and MMR-proficient (HCT116+chr3 and SW480) cell lines, MMR-proficient cells show significantly better survival. These results suggest that the MMR system might be one of the targets of the action of aspirin, and that the MMR system might serve to signal the inhibition of cell growth either directly by promoting apoptosis or indirectly by interfering with the cell cycle progression. These findings additionally strengthen the argument that the antiproliferative effects of aspirin might be independent of its ability to inhibit PG synthesis (5, 26). In fact, we did not detect COX-2 mRNA or protein expression in any of the three cell lines used in this study (data not shown), supporting the role of COX-2-independent effects of aspirin.
The DNA MMR system consists of a complex of proteins that recognizes and directly repairs nucleotide base mismatches and slippage errors at simple repetitive sequences, termed microsatellites (27). The human MMR system also recognizes certain DNA adducts, participates in transcription-coupled DNA repair (28, 29), and regulates the cell cycle (30).

It was shown recently that aspirin and sulindac can suppress the microsatellite unstable mutator phenotype, suggesting that aspirin might provide an effective preventive treatment in hereditary nonpolyposis colorectal carcinoma (13), although the mechanistic explanation of these observations on specific MMR components were not studied. In this study we observed an increase in the expression of key MMR proteins in HCT116+chr3 and SW480 cells. Increased expression of MMR proteins after aspirin treatment is relevant because the MMR system plays a role in regulating the response to mutational damage and in triggering programmed cell death (30, 31). It has been reported that overexpression of the hMLH1 or hMSH2 proteins can induce apoptosis in MMR-deficient or -proficient human colon and endometrial cancer cell lines (32). A number of studies have shown that aspirin and other NSAIDs induce apoptosis in vitro (5, 33) and in vivo (34). These findings are substantiated in our study, in which we observed an increase in apoptosis in HCT116+chr3 and SW480 cells after treatment with aspirin for 48 and 72 h. An interesting feature of these results is the disparate nature of apoptosis induction by aspirin in MMR-proficient and -deficient cells at 48 and 72 h, respectively. Although aspirin caused apoptosis in all of the three cell lines at each concentration and time point, the apoptotic cell population in MMR-proficient cells showed a small decrease after 72 h when compared with 48 h of treatment. On the other hand, in HCT116 cells, the apoptotic cell population showed a small increase at 72 h, suggesting that apoptosis might be an early event in SW480 and HCT116+chr3 cells. When comparing HCT116 and HCT116+chr3 cell lines, it was interesting to observe that both cell lines responded with significantly different rates of apoptosis in response to aspirin treatment, which suggests a possible role for the DNA MMR system in mediating survival of these cells. This finding is consistent with our observation that increased expression of most MMR proteins was observed in only the MMR-proficient HCT116+chr3 and SW480 cells, and suggests the possibility of a MMR-mediated apoptotic effect. Conceivably, aspirin might directly or indirectly regulate the survival of colon cancer cells by interacting with MMR proteins, which are believed to be involved in signaling apoptosis and regulating the cell cycle.

Aspirin arrested HCT116 cells at G0/G1, which is consistent with previous findings using related NSAIDs in different cell models (35, 36). HCT116+chr3 and SW480 cells did not show significant differences in the distribution of cells in various cell cycle phases after aspirin treatment at any concentration or time point, signifying that cell cycle regulation might not be a key mechanism of growth retardation in response to aspirin. These findings are of interest considering the fact that only HCT116 cells showed G0/G1 cell cycle arrest, although these cells lack MMR activity, implying that there are probably two different mechanisms of cell death operative in MMR-deficient and -proficient cells. We hypothesize that growth inhibition observed by aspirin in HCT116 cells is essentially achieved by prolonged cell cycle arrest leading to eventual reproductive cell death. On the contrary, similar treatment with aspirin in MMR-proficient cells responds by up-regulated MMR activity, which results in an increased degree of apoptosis. This strongly suggests that aspirin may be useful as a chemopreventative drug of choice in colorectal cancers with deficient or proficient DNA MMR status.

The observed effects for the action of aspirin on the expression of MMR proteins, apoptosis, and cell cycle were greatest when the cells were treated with low (1 or 2.5 mM) aspirin concentrations. The usefulness of the data presented here is strengthened by the fact that these concentrations of aspirin correspond to salicylate levels measured in the plasma (between 0.5 and 3 mM) of human subjects, as well as to the therapeutic concentrations used in the treatment of arthritis (10, 37). Epidemiological data indicate that aspirin may be chemopreventative when taken at a low dose, and other studies indicate that aspirin has unusual accumulation characteristics in which serum levels increase disproportionately after repetitive administration (38). Therefore, regarding the effective concentrations of aspirin used in our study, the bioavailability of aspirin might result in higher concentrations in intestinal contents, and might be comparable and achievable in a chemopreventative study.

In summary, the experimental data presented here show that aspirin inhibits the growth of colon cancer cells and that the degree of this inhibition is dependent on the MMR status. Defining the key elements of the antitumor effects of aspirin described here will help advance our understanding of these chemopreventative agents and facilitate the development of optimal dosages for clinical trials.

ACKNOWLEDGMENTS
We thank Dennis Young (University of California San Diego Cancer Center Core Facility) for help with flow cytometry analyses and Pamela S. Edmunds for editorial assistance.

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