Mesalazine Improves Replication Fidelity in Cultured Colorectal Cells

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Abstract

Epidemiologic studies indicate that mesalazine has chemopreventive effects in inflammatory bowel disease–associated colorectal cancer. Most of our general understanding of chemoprevention in colorectal cancer is, however, derived from aspirin, which is structurally similar to mesalazine. Herein we determined the influence of aspirin and mesalazine on replication fidelity in cultured colorectal cells. Flow cytometry was used for quantitation of mutation rates at a (CA)₁₃ microsatellite in HCT116 cells (mismatch repair deficient) and HCT116+c.hr3 cells (mismatch repair proficient) that had been stably transfected with pIREShyg2-EGFP/CA13, an enhanced green fluorescence protein–based plasmid, and cultured in the absence or presence of various concentrations of aspirin or mesalazine. Aspirin at doses above 1.25 mmol/L markedly reduced cell growth. Mesalazine doses up to 5.0 mmol/L had no such effect. The mutation rate in mismatch repair–deficient HCT116 cells was $6.8 \times 10^{-4} \pm 9.0 \times 10^{-5}$. In aspirin-treated cultures the mutation rate was $8.2 \times 10^{-4} \pm 1.3 \times 10^{-4}$ (121% of control). Instead, mesalazine lowered the mutation rate in a dose-dependent fashion ($5.5 \times 10^{-4} \pm 1.1 \times 10^{-4}$; 81% of control). The effects of mesalazine were most significant in the M₁ mutation rate, consistent with the mutator phenotype hypothesis as origin of cancer (6). Aspirin increases mismatch repair protein expression and subsequent apoptosis, suggesting that the up-regulation of the mismatch repair system might be another chemopreventive mechanism of aspirin and related nonsteroidal anti-inflammatory drugs (7). We recently developed a flow cytometry–based assay to study replication fidelity. Frameshift mutations were quantified at a (CA)₁₃ microsatellite that shifted an enhanced green fluorescence protein (EGFP) into a +2 position, thereby leading to expression of a truncated nonfluorescent peptide (8). With this assay, we detected three cell populations according to their fluorescence intensity: nonfluorescent, nonmutant M₀ cells; dim fluorescent, intermediate mutant M₁ cells; and strong fluorescent, definitive mutant M₂ cells (9). We showed that intermediate mutant M₁ cells actually carry (CA)₁₂(GT)₁₂ DNA heteroduplexes that are only present immediately after the polymerase error. Failure of mismatch repair resulted in generation of definitive mutant M₂ cells that carry (CA)₁₂(GT)₁₂ DNA homoduplexes. Herein we applied this assay to test a possible effect of aspirin and mesalazine on replication fidelity in cultured colorectal cells.

Introduction

Colorectal cancer is a serious complication in patients with ulcerative colitis or Crohn's colitis. Early age at diagnosis, the extent and severity of colonic disease, the presence of primary sclerosing cholangitis, and a family history of cancer represent independent risk factors for the development of colorectal cancer in ulcerative colitis. The discontinuation of 5-aminosalicylic acid (mesalazine, the active compound of sulfasalazine) therapy was also associated with a higher colorectal cancer risk (1), which was in line with previous findings on sulfasalazine (2, 3), a conjugate of 5-aminosalicylic acid and sulfapyridine. Most of our current understanding of chemoprevention in colorectal cancer is, however, derived from aspirin, acetylsalicylic acid, which is structurally similar to mesalazine. The molecular mechanisms by which aspirin and other nonsteroidal anti-inflammatory drugs exert chemopreventive effects in colon cancer are complex and a matter of ongoing debate. Besides the well-established effects on cyclooxygenase, aspirin and nonsteroidal anti-inflammatory drugs are thought to mediate their antineoplastic properties by other mechanisms including inhibition of nuclear factor κB (4). Another possible target for the activity of nonsteroidal anti-inflammatory drugs is the improvement of DNA replication. The fidelity of DNA replication is a product of polymerase accuracy, its proofreading activity, and the proficiency of the postreplication mismatch repair system (5). Inefficiency of one of these processes can be a key to the development of human cancer, best illustrated by the familial cancer syndrome hereditary nonpolyposis colorectal cancer (also called Lynch syndrome). In hereditary nonpolyposis colorectal cancer, loss-of-function mutations of DNA mismatch repair proteins, such as hMLH1 or hMSH2, reduce the activity of postreplication DNA mismatch repair and strongly elevate the mutation rate, consistent with the mutator phenotype hypothesis as origin of cancer (6). Aspirin increases mismatch repair protein expression and subsequent apoptosis, suggesting that the up-regulation of the mismatch repair system might be another chemopreventive mechanism of aspirin and related nonsteroidal anti-inflammatory drugs (7). We recently developed a flow cytometry–based assay to study replication fidelity. Frameshift mutations were quantified at a (CA)₁₃ microsatellite that shifted an enhanced green fluorescence protein (EGFP) into a +2 position, thereby leading to expression of a truncated nonfluorescent peptide (8, 9). With this assay, we detected three cell populations according to their fluorescence intensity: nonfluorescent, nonmutant M₀ cells; dim fluorescent, intermediate mutant M₁ cells; and strong fluorescent, definitive mutant M₂ cells (9). We showed that intermediate mutant M₁ cells actually carry (CA)₁₂(GT)₁₂ DNA heteroduplexes that are only present immediately after the polymerase error. Failure of mismatch repair resulted in generation of definitive mutant M₂ cells that carry (CA)₁₂(GT)₁₂ DNA homoduplexes. Herein we applied this assay to test a possible effect of aspirin and mesalazine on replication fidelity in cultured colorectal cells.

Materials and Methods

Cell culture. Details of the in vitro bioassay to study mutation rates at a (CA)₁₃ repetitive sequence have been previously described (9). In brief, a (CA)₁₂(GT)₁₂ oligonucleotide was inserted after the translation initiation codon of the EGFP gene of the plasmid pIREShyg2-EGFP resulting in the plasmid pIREShyg2-EGFP/CA13. In this construct, the downstream portion of the EGFP gene had been shifted out of its reading frame, resulting in expression of a truncated missense peptide.
without fluorescence. Deletion of 2 bp within this dinucleotide repeat shifts the EGFP gene into the proper reading frame and causes expression of EGFP. The pREShyg2-EGFP/CA13 construct had been stably transfected into HCT116 colorectal cancer cells (ATCC no. CCL-247), which are mismatch repair deficient by homozygous mutation of the hMLH-1 gene, and HCT116-c3h, which are mismatch repair proficient through transfer of chromosome 3 (10). For all experiments, well-characterized single cell clones from HCT116 cells (clones A1.3 and A2.1) and from HCT116-c3h cells (clones A3.1, A3.3, and A3.7) were used. Cells were grown in Iscove’s modified Dulbecco’s medium (Life Technologies Inc., Rockville, MD) as described (9).

Mesalazine (generously provided by Dr. Falk Pharma, Freiburg, Germany) and aspirin (purchased form Sigma Chemical Co., St. Louis, MO) were dissolved in DMSO and sterile filtered. Twenty-four hours after initial cell sorting, mesalazine or aspirin was added to quadruplicate cultures at a final concentration of 0.0 to 5.0 mmol/L. Corresponding concentrations of DMSO were used as control. All experiments were done at least twice per clone.

A flow cytometry–based assay for mutagenesis. One thousand nonfluorescent cells were sorted into 24-well plates on a FACSVantage SE using CloneCyt Plus sorting technology (Becton Dickinson Immunocytometry Systems, San Jose, CA). After an 8-day growth period at 37°C, 5% CO2, and full humidity, cells were trypsinized, washed in PBS containing 2% fetal bovine serum (FBS), and resuspended in a total volume of 100 μL PBS/2% FBS. Fifty microliters of cell suspension were analyzed on a FACSCalibur with CellQuest acquisition and analysis software (Becton Dickinson) and the cell counts were doubled to quantitate the total cell number per well. The counts of low fluorescent M1 cells (intermediate mutants), and high fluorescent M2 cells (definitive mutants) were expressed as fractions of the total cell number in culture. Mutation rates were calculated as previously described (9, 11).

Statistics. Means and SEs were calculated for continuous variables (e.g., fluorescence intensity). Cochran-Armitage trend tests were used to compare mutant fractions across different doses of aspirin or mesalazine. The mutation rate is defined as the probability of a cell undergoing a mutation in its lifetime, and is expressed per microsatellite per cell per generation. Mutation rates were estimated using two methods: the method of the mean and the maximum likelihood method (11). A single mutation rate for each dose was estimated by taking a weighted linear combination of the dose-specific mutation rates across clones, with weights chosen to be inversely proportional to the individual variances. All estimated quantities are presented with SEs based on quadruple cultures for each clone.

Results

Effect of aspirin and mesalazine on cell viability. We chose to test aspirin and mesalazine for their effect on frameshift mutations in human mismatch repair–deficient colorectal cancer cells. Various concentrations of aspirin and mesalazine were analyzed regarding cell growth. At aspirin concentrations above 2.5 mmol/L, cells started to detach from the bottom of the culture plate within 24 hours. This observation was accompanied by a significant reduction of the total cell number for any aspirin concentration above 1.25 mmol/L at the end of the culture period (Fig. 1). Mesalazine treatment up to 5.0 mmol/L displayed no such effect.

Mesalazine reduces the mutation rate in mismatch repair–deficient cells. The effects of aspirin and mesalazine on mutation rates were further analyzed for any nontoxic dose (aspirin ≤ 1.25 mmol/L, mesalazine ≤ 5.0 mmol/L). Mesalazine, but not aspirin, reduced the mutant fraction in culture in both HCT116-A1.3 and HCT116-A2.1 clones (Fig. 2). Cochran-Armitage trend tests revealed significant decreases in mutant fractions as the dose of mesalazine increased, with χ² statistics of 141.91 (P < 0.0001) and 106.70 (P < 0.0001) for clones A1.3 and A2.1, respectively. For aspirin the trend test was not significant for either clone ($\chi^2$ statistic = 1.25, $P = 0.26$ for A1.3; $\chi^2$ statistic 1.66, $P = 0.20$ for A2.1).

Figure 1. Effect of aspirin and mesalazine on the number of cultured cells. Twenty-four hours after sorting 1,000 cells into 24-well plates, various concentrations of aspirin and mesalazine were added to HCT116-A2.1 cells. Cells were cultured for another 7 days, harvested, and finally the total cell count was analyzed by flow cytometry. A dose-dependent reduction in the total cell count was seen for any aspirin concentrations above 1.25 mmol/L but not for mesalazine up to 5.0 mmol/L.
Mesalazine improves replication fidelity in mismatch repair–proficient cells. Next, we tested the effect of mesalazine in mismatch repair–proficient HCT116+chr3 clones. As previously observed (9), the intermediate mutant M1 cell fraction in HCT116+chr3 clones (0.22 ± 0.05%) was somewhat smaller than in HCT116 clones (0.35 ± 0.05%). HCT116+chr3 cells also did not generate a sizeable number of definitive mutant M2 cells within this short period of time. Therefore, we were unable to measure an effect of mesalazine on generation of M2 cells. Mesalazine decreased the number of M1 cells in a dose-dependent manner (Fig. 3, P < 0.0001). Aspirin or DMSO had no effect on the generation of M1 cells. The effect of mesalazine was also seen when mesalazine was dissolved in ethanol instead of DMSO, showing that the prevention of mutations is an effect of mesalazine and not of the solvent.

Discussion

Herein we present experiments that suggest an effect of mesalazine on the occurrence of frameshift mutations at a (CA)13 microsatellite, which is quite different from the chemopreventive effects of aspirin despite the structural similarities. The findings have been generated in a flow cytometry–based assay using two near isogenic human colorectal cell lines. HCT116 cells originate from a microsatellite instability–positive colorectal cancer from a hereditary nonpolyposis colorectal cancer patient and do not express hMLH1 (12). In HCT116+chr3, the hypermutable phenotype of HCT116 cells has been reversed through transfer of chromosome 3 that encodes a wild-type hMLH1 gene (10). In fact, the mutation rate at the (CA)13 microsatellite in HCT116+chr3 cells is 30 times lower than in

![Figure 2](image-url)

**Figure 2.** Effects of aspirin and mesalazine on the mutation rate at a (CA)13 microsatellite. Nonfluorescent HCT116 cells have been sorted into 24-well plates (1,000 cells/well) and cultured for 8 days with addition of various nontoxic concentrations of aspirin (0-1.25 mmol/L) and mesalazine (0-5.0 mmol/L). Cells were harvested and fluorescent (mutant) cells were quantitated by flow cytometry as described (9). The mutant fraction was calculated as the number of fluorescent cells (M1 and M2) per total cells. Treatment with mesalazine (B), but not with aspirin (A), caused a dose-dependent drop in the mutant cell fraction (P < 0.0001 for mesalazine). Points, mean of quadruple cultures for each clone (HCT116-A1.3 and HCT116-A2.1); bars, SE.

![Figure 3](image-url)

**Figure 3.** Differential effect of mesalazine on the intermediate mutant (M1) and definitive mutant (M2) population. Subanalyses of the experiments in HCT116 cells testing mesalazine (as described in Fig. 2B) were done. The mutant fraction was separately expressed for M1 cells (A) and M2 cells (B). The effect of mesalazine was greatest for intermediate mutant M1 cells (i.e., early mutation events immediately after the initial replication error, P < 0.0001; A). The mesalazine-associated reduction of intermediate mutant M1 cells was followed by a drop in definitive mutant M2 cells at higher mesalazine concentration (P < 0.0001; B). A dose-dependent drop in the intermediate mutant M1 cell fraction was also observed in mismatch repair–proficient HCT116+chr3 (P < 0.0001; C). A similar drop was seen when mesalazine was dissolved in ethanol (HCT116+chr3-A3.1) instead of DMSO (HCT116+chr3-A3.3 and HCT116+chr3-A3.7). Due to mismatch repair proficiency, HCT116+chr3 cells did not generate a sizeable number of definitive mutant M2 cells. Therefore, the effect of mesalazine on generation of definite mutant M2 cells was not tested in HCT116+chr3.
from stably transfected cells, however, as used herein, H2O2 had similar model based on transient transfections (8). In clones H2O2, we did not test any protective effect of mesalazine in the genome. Because we were unable to induce mutations by this is due to protection of plasmid DNA when integrated into cultures with H2O2.

Frameshift mutations at microsatellites occur as a time-dependent function of polymerase errors followed by failure of postreplication mismatch repair. Because the effect of mesalazine was seen in mismatch repair–deficient HCT116 cells and in intermediate mutant M1 cells, mesalazine may act on replication fidelity independent of the postreplication mismatch repair. Numerous processes determine the fidelity of DNA replication besides mismatch repair. Several of these are responsible for providing undamaged substrates to the replication machinery (e.g., sanitizing deoxynucleotide triphosphate pools, base excision repair, nucleotide excision repair, and single-strand DNA break repair; ref. 13). Next, replication accuracy of undamaged DNA depends on the high nucleotide selectivity and proofreading activity of the various polymerases, which is best illustrated by the emerging relationships between DNA polymerase dysfunction and cancer (13).

Previously we induced frameshift mutations by H2O2 in a similar model based on transient transfections (8). In clones from stably transfected cells, however, as used herein, H2O2 had no effect on the mutation rate (data not shown). It is likely that this is due to protection of plasmid DNA when integrated into the genome. Because we were unable to induce mutations by H2O2, we did not test any protective effect of mesalazine in cultures with H2O2.

Table 1. Mutation rates at a (CA)13 microsatellite for two mismatch repair–deficient HCT116 cell clones estimated by two different computational methods

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Method of the mean</th>
<th>Maximum likelihood</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HCT116-A1.3</td>
<td>HCT116-A2.1</td>
</tr>
<tr>
<td>None</td>
<td>8.9 ± 1.8 (100%)</td>
<td>7.1 ± 2.1 (100%)</td>
</tr>
<tr>
<td>Mesalazine</td>
<td>6.7 ± 1.8 (75%)</td>
<td>5.0 ± 2.6 (70%)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>9.6 ± 2.3 (108%)</td>
<td>7.7 ± 3.0 (108%)</td>
</tr>
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NOTE: Data × 10^-4 are mean ± SE of mutation rates per microsatellite per generation. Both drugs were used at the highest nontoxic dose (mesalazine at 5 mmol/L and aspirin at 1.25 mmol/L).

HCT116 (9). When transferring our experimental finding from these cell lines into models of human cancer, we would expect that mesalazine could improve replication fidelity and thereby reduce the tempo and frequency of cancer development. Clinical studies have indeed shown that mesalazine and its predecessor sulfasalazine reduce the risk of colorectal cancer in ulcerative colitis (1–3).

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F 4.0 (100%) 7.6 1.3 (100%) 5.2 1.2 (100%) 7.3 1.2 (100%) 5.3 1.1 (100%) 7.1 1.1 (100%)

The observed effects of mesalazine on replication fidelity were seen at mesalazine concentrations above 1.25 mmol/L (HCT116-A1.3) or 2.5 mmol/L (HCT116-A2.1) and were greatest at the highest dose tested (5.0 mmol/L). Similar or higher mesalazine concentrations were applied in other experimental settings and measured in the colon of patients with ulcerative colitis (19). Mesalazine microgranules reached concentrations above 1 mmol/L in the ileal fluid (20). Because 90% of the oral dose are delivered to the colon, extrapolation of these measurements reveals a colonic mesalazine concentration above 10 mmol/L, which is clearly beyond the highest concentration in our experiments (20).

At the 5.0 mmol/L level, a 19% reduction of the mutation rate was observed in mismatch repair–deficient HCT116. Considering the fact that the tempo of mutations defines the speed of tumor progression (as predicted in the mutator phenotype hypothesis ref. 6), mesalazine therapy could introduce a significantly delay in the clinical manifestation of a tumor. If mesalazine increases the number of years it takes for cells to accumulate the requisite number of mutations required for invasiveness or metastases by 19%, it would significantly reduce the life-threatening manifestations of cancer and diminish cancer deaths even in the absence of reducing cancer incidence. In this respect, mesalazine is potentially useful for prevention of colorectal cancer independent of its anti-inflammatory properties.

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References
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