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 quantification of mutation rates at a (CA)13 microsatellite in HCT116 cells (mismatch repair deficient) and HCT116+chr3 cells (mismatch repair proficient). The effects of aspirin on replication fidelity were most significant in the M1 (CA)13 microsatellite in cultured colorectal cells independent of mismatch repair proficiency. This finding suggests that mesalazine improves replication fidelity, an effect that may be active in reducing mutations independent of its anti-inflammatory properties.

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)12 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)12 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 pIREShyg2-EGFP/CA13 construct had been stably transfected into HCT116 colorectal cancer cells (ATCC no. CCL-247), which are mismatch repair deficient by homoygous mutation of the hMLH-1 gene, and HCT116-ch3, 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-ch3 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 fluorescence 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 but not for mesalazine up to 5.0 mmol/L.

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). This reduction in mutant cells by mesalazine was mainly caused by a decrease in intermediate mutant M1 cells (Cochran-Armitage test statistics of 159.45 and 103.88 with associated $P$ values of <0.0001 for the clones A1.3 and A2.1, respectively) and also by a reduction in definitive mutant M2 cells (Cochran-Armitage test statistics of 23.59 and 18.71 with associated $P$ values of <0.0001 for the clones A1.3 and A2.1, respectively; Fig. 3). No change in the mutant fraction was observed when cells were cultured in the solvent alone. The combined (across clones) mutation rate dropped from $6.8 \times 10^{-4} \pm 9.0 \times 10^{-5}$ to $5.5 \times 10^{-4} \pm 1.1 \times 10^{-4}$ (81% of control; Table 1). No such change or, rather, an increase was estimated for aspirin ($8.2 \times 10^{-4} \pm 1.3 \times 10^{-4}$; 121% of control; Table 1).

Because the effect was mainly caused by a reduction in M1 cells, we wanted to exclude methodologic errors such as an effect of mesalazine on EGFP fluorescence. HCT116 cells were stably transfected with pIREShyg2-EGFP, a plasmid that was identical to pIREShyg2-EGFP/CA13 but lacking the (CA)$_{13}$ microsatellite, and expressed EGFP within the reading frame. EGFP-expressing cells were cultured with mesalazine and the mean fluorescence intensity was measured by flow cytometry. The mean fluorescence intensity between different cultures ranged from 374 to 448 and was not different between the various mesalazine concentrations and the blank (data not shown). It is therefore unlikely that mesalazine interferes with EGFP fluorescence.

We previously have shown that M2 cells display a deletion of one CA-dinucleotide within the microsatellite (9). The reduction in M2 cells therefore reflects a reduction in the microsatellite mutation rate by mesalazine. Because HCT116 cells are mismatch repair deficient, this effect can only be explained by mechanisms independent of mismatch repair, such as an improvement of replication fidelity. Indeed, mesalazine significantly lowered the number of intermediate mutant M1 cells, which reflects a population of cells immediate after the polymerase error (Fig. 3B). The mutation-protective effect of mesalazine is therefore caused by improvement of polymerase fidelity rather than by mismatch repair fidelity.

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 \( \pm \) 0.05\%) was somewhat smaller than in HCT116 clones (0.35 \( \pm \) 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. 3C, \( P < 0.0001 \)). 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. 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.

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 \( \pm \) 0.05\%) was somewhat smaller than in HCT116 clones (0.35 \( \pm \) 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. 3C, \( P < 0.0001 \)). 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
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).

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 (14).

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 this is due to protection of plasmid DNA when integrated into no effect on the mutation rate (data not shown). It is likely that the type of polymerase. Frameshift fidelity also 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 (14).

Currently, we do not understand the molecular mechanism of how mesalazine interferes with the generation of frameshift mutations. We also do not know whether this effect is only true for poly-CA tracts or also for other repetitive sequences. The best known model of frameshift mutations at repetitive sequences involves strand misalignment through template-primer slippage (reviewed in ref. 15). Slippage depends on various factors such as the length of the repetitive sequence, the type of nucleotide (pyrimidine runs show higher slippage rates that purine runs), and the type of polymerase. Frameshift fidelity also decreases when proofreading is suppressed or when the composition of the nucleotide pool is changed (16). Because most control mechanisms of replication affect not only frameshift mutations but also base substitution fidelity, we may speculate that mesalazine might also prevent missense mutations (5).

The reduction in cell number by aspirin was mainly caused by cell death (17). Mesalazine also reduced cell numbers at the highest concentration tested but rather by growth inhibition. This is in line with previous studies showing accumulation of cells in the S and G2-M phases of the cell cycle (18). The lower number of mutant cells, however, cannot be explained by simple growth retardation because we measured the mutant cells as fraction of the total cell number at the end of the culture period. However, we cannot exclude that improved replication accuracy takes time and thus may delay cell cycle progression.

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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**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>6.0 ± 1.1 (100%)</td>
</tr>
<tr>
<td>Mesalazine</td>
<td>6.7 ± 1.8 (75%)</td>
<td>4.9 ± 1.4 (82%)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>9.6 ± 2.3 (108%)</td>
<td>7.8 ± 1.6 (130%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>HCT116-A1.3</th>
<th>HCT116-A2.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.1 ± 2.1 (100%)</td>
<td>4.8 ± 1.1 (100%)</td>
</tr>
<tr>
<td>Mesalazine</td>
<td>5.0 ± 2.6 (70%)</td>
<td>4.4 ± 2.1 (92%)</td>
</tr>
<tr>
<td>Aspirin</td>
<td>7.7 ± 3.0 (108%)</td>
<td>7.4 ± 2.1 (154%)</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).
References

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