Sulindac Inhibits β-Catenin Expression in Normal-Appearing Colon of Hereditary Nonpolyposis Colorectal Cancer and Familial Adenomatous Polyposis Patients

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Abstract

Sulindac reduces colorectal cancer risk in genetically susceptible humans and animals. The molecular mechanisms underlying these effects are incompletely understood. Many studies suggest an important role for induction of apoptosis involving the mitochondrial pathway and the death receptor pathway. Alternatively, mechanisms involving the APC-β-catenin-Wnt pathway have been suggested, possibly mediated by p21. We determined the effects of sulindac on apoptosis and expression of death receptor (DR)-4 and DR5, β-catenin, and p21 in normal-appearing colorectal epithelium. Biopsies were obtained before and after sulindac treatment during two chemoprevention studies. Patients (n = 18) with hereditary nonpolyposis colorectal cancer (HNPPC) received 150 mg sulindac bd for 4 weeks in a placebo-controlled crossover design. Patients (n = 6) with familial adenomatous polyposis (FAP) received 150 mg sulindac bd for 6 months. Apoptosis was assessed by M30 staining and expression patterns of DR4, DR5, β-catenin, and p21 were studied immunohistochemically. In HNPPC patients, apoptotic indices were similar following placebo and sulindac. Also in FAP patients, apoptotic indices were not different after sulindac compared with pretreatment values. Expression of DR4 and DR5 was observed in all samples with no consistent differences between placebo/baseline and sulindac. Intensity of membranous β-catenin staining was lower in HNPPC samples following sulindac compared with placebo (P < 0.001). Similar results were obtained in FAP samples (P < 0.01). p21 expressions before and after sulindac treatment were similar in both patient groups. In conclusion, sulindac inhibits β-catenin expression in normal colorectal epithelium from HNPPC and FAP patients without affecting apoptotic indices and DR4, DR5, and p21 expression.

Introduction

Colorectal cancer is the second leading cause of cancer death in the Western world. Familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC) are well-defined conditions predisposing to colorectal cancer (1). Numerous studies have established the chemopreventive effects of nonsteroidal anti-inflammatory drugs as sulindac (2-4) and celecoxib (5) in patients with FAP, whereas studies in HNPCC patients are ongoing (6).

The precise mechanisms by which nonsteroidal anti-inflammatory drugs mediate their effects are incompletely understood, but likely involve induction of apoptosis (7-9). Apoptosis is controlled via two major pathways, one originating at the cell membrane, the extrinsic pathway, and one involving the mitochondria, the intrinsic pathway (10). Apoptotic pathways originating at the cell membrane involve death receptors like Fas, tumor necrosis factor receptor 1, death receptor (DR)-4, DR4, and DR5, which are activated on binding to their respective ligands (10). Recent reports reveal that the nonsteroidal anti-inflammatory drug sulindac mediates apoptosis through the mitochondrial pathway in colon cancer cells, involving caspase 9 and BAX (11). Sulindac-induced apoptosis has also been shown to involve death receptor DR5 in colon cancer cells (12, 13). In vitro, sulindac administration induced up-regulation of DR5 mRNA and protein levels, but not of DR4 (12, 13).

The initial event in the neoplastic transformation of normal colon epithelium is assumed to be the activation of the Wnt signaling pathway, caused by mutations in the APC or the β-catenin gene (14). This leads to cytoplasmic and subsequent nuclear accumulation of β-catenin. In the nucleus, β-catenin binds and activates the transcription factor T-cell factor 4. Finally, activated T-cell factor 4 activates a genetic program presumed to be responsible for early adenoma formation (14).

Several in vitro studies suggest that sulindac mediates its antineoplastic effect by inhibition of the Wnt pathway (15-17). This is supported by studies in APCmin mice (18) and in adenomas of FAP patients (15). Recent reports reveal that sulindac affects Wnt signaling by modifying expression of p21, a cyclin-dependent kinase inhibitor (19).

To provide further insight into the mechanisms involved in the chemopreventive action of sulindac, we investigated the effects on apoptosis and expression of DR4, DR5, β-catenin, and p21 in normal epithelium of FAP and HNPPC patients.

Materials and Methods

Patient Selection. Recently, a chemoprevention biomarker study was done in proven or probable HNPPC patients at the University Medical Center Groningen (20). Proven patients were carriers of a mutation in one of the mismatch repair genes (hMLH1, hMSH2, hMSH6). Probable HNPPC patients had a
family history meeting the revised Amsterdam criteria (21) and a medical history of an HNPCC-associated cancer, a colorectal adenoma at an early age (<40 years), or an adenoma with advanced neoplastic characteristics. Individuals with prior colorectal surgery were enrolled when the estimated length of the remaining colon exceeded 50% of the original length. In this randomized double-blind placebo-controlled crossover study, patients were assigned to receive sulindac 150 mg orally twice daily or an identically appearing placebo for 4 weeks. Both were produced by the Pharmacy Department of the University Medical Center. After a washout period of 4 weeks, patients crossed over to the alternative treatment for another 4 weeks. Full colonoscopy was done at 4 and 12 weeks. Biopsies were taken of macroscopically normal mucosa with a standardized biopsy forceps at four locations: ascending, transverse, and sigmoid colon, and rectum. Samples were formalin fixed, embedded in paraffin, and coded to disguise the subjects’ treatment assignment. The local medical ethical committee approved the study. Reasons for exclusion from the study were use of a nonsteroidal anti-inflammatory drug in the 3 months before the study, pregnancy, or a history of peptic ulcer disease or gastrointestinal bleeding.

For the present study, sufficient residual material was available from 18 patients (12 men, 6 women; mean age, 44.6 years). Nine of these patients were proven carriers of a mutation in the mismatch repair gene hMSH2 (n = 6) or hMLH1 (n = 3).

Samples from FAP patients were obtained from a study in which FAP patients had been treated with sulindac 150 mg twice daily during 9 months, as described previously (2, 22). From six patients, tissue sections were available from normal-appearing rectal mucosa before and after 6 months of treatment. These six patients had adenomas at baseline and showed regression of adenomas after treatment with sulindac.

**Immunohistochemistry for Apoptosis, DR4, DR5, β-Catenin, and p21.** For immunohistochemistry, 3 μm thick sections were cut from paraffin blocks and deparaffinized in xylene. Apoptosis was determined using the murine monoclonal antibody M30 (Boehringer Mannheim, Mannheim, Germany) directed against cleaved cytokeratin-18 that is expressed during early apoptosis (23). Staining procedures for M30, DR4, and DR5 were done as previously described (23, 24). For β-catenin staining (1:1,000; clone 14, Transduction Laboratories, Lexington, KY), antigen retrieval was carried out by microwave treatment for 8 minutes at 700 W in 0.01 mol/L citrate buffer (pH 6.0). For p21 staining (1:50; clone WAF1, Oncogene Research, Darmstadt, Germany), antigen retrieval was done by heating slides thrice for 5 minutes at 115°C with 5-minute cooling in between in maleate buffer in a preheated autoclave ( Presto deluxe, Presto, Eclaire, WI). After blocking of endogenous peroxidase with 0.3% hydrogen peroxide for 30 minutes and incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA), primary antibodies were applied for 1 hour at room temperature. After washing with PBS, slides were incubated with appropriate secondary and tertiary antibodies. Slides were counterstained with hematoxylin. As negative controls, slides were stained in absence of the primary antibody. As positive controls, sections of normal human liver (DR4 and DR5) and colorectal cancer (β-catenin, p21, and M30) were included. For each antibody, slides were stained in one batch.

**Evaluation of Staining.** Slides were independently evaluated by light microscopy by two investigators in a coded fashion. For M30 and p21, positive cells were expressed as percentage of the total number of cells counted (apoptotic and p21 indices, respectively). Only completely longitudinal crypts and at least 500 cells were counted. Intensity of DR4, DR5, and β-catenin staining was semiquantitatively graded using a scale from 1 to 3 (1, weak staining; 2, moderate staining; 3, intense staining). For β-catenin, staining was separated recorded as membranous, cytoplasmic, or nuclear. To assess changes in staining intensity as a consequence of treatment, intensities were compared in paired slides and scored as increased, decreased, or unchanged. When the observers’ scores differed, cases were reevaluated using a multheaded microscope and the final grade was reached by consensus.

**Results**

To assess changes in apoptosis, DR4, DR5, β-catenin, and p21 expression following placebo and sulindac, samples were analyzed pairwise, comparing staining results in biopsies from the same patient in the same colonic region. The analysis of sample pairs was hampered by the problem of limited availability of material. In case one in a pair of samples contained insufficient material to allow evaluation, it meant that these samples were not evaluated. Not all biopsies obtained in HNPCC patients were of sufficient quality, limiting the number of sample pairs analyzed to 55 for apoptotic indices, 64 for DR4, 67 for DR5, 48 for β-catenin, and 63 for p21 expression. The number of sample pairs analyzed from FAP patients was six for all staining procedures.

**Apoptosis.** When comparing cumulative apoptotic indices between placebo and sulindac treatment (in HNPCC) and between pretreatment and posttreatment with sulindac (in FAP), no statistically significant differences were observed (Table 1, left). Given the predilection for the proximal colon in the development of colorectal neoplasia in HNPCC, apoptotic indices were compared in different colonic regions in HNPCC patients. For each region, apoptotic indices were not significantly different following sulindac compared with placebo although in biopsies from the proximal colon there was a trend towards lowering of apoptotic indices following sulindac.

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**Statistics.** For statistical assessment of changes in apoptotic indices, p21 indices, and cumulative DR4, DR5, and β-catenin expression scores following sulindac treatment versus placebo, the Wilcoxon rank sum test for paired samples was used. Changes in distribution of staining intensities of DR4, DR5, and β-catenin were assessed using χ² tests. To determine differences between various colonic regions in HNPCC patients, Mann-Whitney tests for continuous variables and χ² tests for discontinuous variables were conducted. Differences between proven and probable HNPCC patients were assessed using Mann-Whitney test for continuous variables and χ² tests for discontinuous variables. Reported P values were two tailed and significance was assumed at P < 0.05. SPSS for Windows software (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

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**DR4, DR5, and β-Catenin Expression following Placebo (HNPCC) and at Baseline (FAP).** In all patient samples, cytoplasmic staining of DR4 and DR5 was observed. For DR4, the immunoreactivity of epithelial cells increased gradually from the crypt base to the luminal surface. DR5 immunoreactivity was seen along the entire crypt axis. β-Catenin expression was membranous in all investigated samples (i.e., no cases of cytoplasmic or nuclear staining were seen). In HNPCC patients, DR4, DR5, and β-catenin staining intensities were similar in all four investigated regions of the colon. No differences were seen between proven carriers of MLH1 or MSH2 gene mutations and patients without an established mutation. Also, no differences in expression patterns were observed between MLH1 and MSH2 mutation carriers.

**Changes in DR4, DR5, and β-Catenin Expression following Sulindac (HNPCC and FAP).** Alterations in expression patterns of DR4, DR5, and β-catenin were analyzed, studying the distribution of staining intensities and changes in absolute and cumulative staining intensity scores. To assess whether
changes in staining intensities were consistent in HNPCC patients, cumulative scores were calculated for each patient by adding the respective intensity scores in the samples from different parts of the colon. Cumulative scores were calculated when at least two sample pairs per patient were available.

Table 2 summarizes changes in the distribution of staining intensities of DR4, DR5, and b-catenin expression following sulindac compared with placebo (HNPCC) and baseline (FAP). For DR4 and DR5, staining scores were similarly distributed following sulindac compared with placebo in HNPCC patients and compared with baseline values in FAP patients. For b-catenin, staining scores were distributed differently following sulindac compared with placebo in HNPCC (P < 0.001) and compared with baseline values in FAP samples (P < 0.01; Table 2), with lower scores in both patient groups after sulindac.

In sample pairs, the intensity scores of DR4 staining were not consistently different following sulindac compared with placebo in HNPCC: higher in 26 of 64 pairs, lower in 27 of 64 pairs, and unchanged in 11 of 64 pairs (not significant). For DR5, similar results were obtained: higher in 19 of 67, lower in 20 of 67, and unchanged in 28 of 67 pairs (not significant). The intensity scores of membranous b-catenin staining following sulindac compared with placebo were higher in 7 of 48, lower in 26 of 48, and unchanged in 15 of 48 pairs (P < 0.05). In paired FAP samples, DR4 and DR5 staining intensities were similar between baseline and sulindac in all six samples. For membranous b-catenin, staining intensities were lower following sulindac in three of six and unchanged in three of six FAP pairs (not significant). In cases with lower staining intensity of membranous b-catenin following sulindac, no apparent increase in cytoplasmic or nuclear staining was seen.

With respect to cumulative staining intensity scores in HNPCC, scores were similar for DR4 and DR5 following placebo and sulindac (data not shown). However, for b-catenin, cumulative intensity scores were significantly lower following sulindac compared with placebo (P < 0.01; Fig. 1).

p21. Mean percentages of p21-positive cells following placebo and sulindac are shown in Table 1 (right). After placebo, p21 indices were comparable in different colon regions in HNPCC patients. p21 indices were not significantly different between HNPCC and FAP patients. Following sulindac, p21 indices were similar compared with placebo (HNPCC) and baseline (FAP) values.

### Discussion

The efficacy of chemopreventive agents in the colorectum is routinely assessed by measuring one or more end points: biomarker modulation in the at-risk mucosa, adenoma regression, adenoma suppression, or adenoma prevention (25). Our biomarker modulation study evaluated changes in apoptosis and expression of DR4, DR5, b-catenin, and p21 occurring in normal-appearing mucosa following treatment with sulindac in HNPCC and FAP patients. Although, in general, few conclusions can be drawn from biomarker studies, they provide an opportunity to identify mechanisms of action of chemopreventive agents. In particular, quantitative measurements of apoptosis are considered a sensitive index of the biological effects of nonsteroidal anti-inflammatory drugs.

### Table 2. Distribution of DR4, DR5, and b-catenin staining intensities in sample pairs from normal colon mucosa following sulindac compared with placebo (HNPCC) and baseline values (FAP)

<table>
<thead>
<tr>
<th>Staining score</th>
<th>HNPCC</th>
<th>FAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Placebo</td>
<td>Sulindac</td>
</tr>
<tr>
<td>n/n tested (%)</td>
<td>n/n tested</td>
<td>n/n tested</td>
</tr>
<tr>
<td>DR4 1</td>
<td>5/64 (8%)</td>
<td>6/64 (9%)</td>
</tr>
<tr>
<td>2</td>
<td>31/64 (48%)</td>
<td>32/64 (50%)</td>
</tr>
<tr>
<td>3</td>
<td>28/64 (44%)</td>
<td>26/64 (41%)</td>
</tr>
<tr>
<td>DR5 1</td>
<td>10/67 (15%)</td>
<td>9/67 (13%)</td>
</tr>
<tr>
<td>2</td>
<td>36/67 (54%)</td>
<td>39/67 (58%)</td>
</tr>
<tr>
<td>3</td>
<td>21/67 (31%)</td>
<td>19/67 (28%)</td>
</tr>
<tr>
<td>b-Catenin 1</td>
<td>4/48 (8%)</td>
<td>9/48 (19%)</td>
</tr>
<tr>
<td>2</td>
<td>24/48 (50%)</td>
<td>33/48 (69%)</td>
</tr>
<tr>
<td>3</td>
<td>20/48 (42%)</td>
<td>6/48 (12%)</td>
</tr>
</tbody>
</table>

*Assessed as described in the Materials and Methods section.

1 Number of samples investigated varied in patient groups as a consequence of limited availability of slides.

2 Distribution of staining intensities of b-catenin in HNPCC following placebo versus sulindac, P < 0.001.

3 Distribution of staining intensities of b-catenin in FAP at baseline versus sulindac, P < 0.01.
(6). Whereas several biomarker modulation studies are available in FAP patients, our placebo-controlled crossover study is one of only a few in HNPCC patients. We found that sulindac did not alter the apoptotic index in normal colorectal mucosa from HNPCC and FAP patients compared with placebo (HNPCC) or baseline (FAP). As anticipated from these null results, no changes were seen in expression of the death receptors DR4 and DR5. However, reduced membranous β-catenin expression patterns were observed following sulindac in both patient groups, suggesting an inhibiting effect of sulindac on the APC-β-catenin-Wnt pathway.

Sulindac is one of the most extensively studied nonsteroidal anti-inflammatory drugs in the setting of chemoprevention of colorectal cancer (7). An important mechanism behind the chemopreventive effect of sulindac seems to be the induction of apoptosis (7). Sulindac is a prodrug that is converted into sulfide and then sulindac sulfone by colonic bacteria (26). In vitro, both metabolites induce apoptosis in colon cancer cells (7, 11, 27), including mismatch repair–deficient cells (28). In APCMin mice, a mouse model of FAP, sulindac had an antitumor effect and was associated with induction of apoptosis (29). Also in a mismatch repair–deficient APCMin mouse model, carrying genetic features of both FAP and HNPCC, sulindac inhibited intestinal adenoma development (30). Whether this effect was mediated by induction of apoptosis was not studied. In normal rectal mucosa of FAP patients with adenomas, an increase or change of apoptosis has been observed following sulindac therapy (8, 31). We did not find a significant effect on apoptosis in our FAP material, but this may be due to the limited number of cases. Interestingly, in presymptomatic, phenotypically unaffected patients with adenomas, an increase or change of apoptosis (32). Whether this effect was mediated by induction of apoptosis, but limited to the stage when adenomas have already developed.

Recent studies have suggested that β-catenin is a target for the chemopreventive action of nonsteroidal anti-inflammatory drugs (16, 35, 36). In vitro, nonsteroidal anti-inflammatory drugs, including sulindac, prevented nuclear accumulation of β-catenin (16, 35). Oncogenic activation of the Wnt signaling pathway resulting in nuclear translocation of β-catenin is considered critical for the initiation in intestinal epithelial neoplastic transformation (37). A recent report reveals that adenomas from FAP patients showed less nuclear β-catenin staining after sulindac treatment (15). Similar results were obtained in APCMin mice, in normal intestinal mucosa (38) as well as in adenomas (39). Our results in normal colon mucosa, with a reduction in membranous expression of β-catenin following sulindac, are consistent with these data. Whether this phenomenon is limited to subjects with a predisposition for colorectal adenoma development or also applies to the general population remains unknown.

Finally, we assessed whether changes in β-catenin expression were associated with alterations in p21 expression. Recent data indicated that active Wnt signaling decreases p21 concentrations, preventing cells from entering G1 arrest or differentiation, thereby allowing cells to proliferate (40). In a previous study of three patients treated with sulindac, p21 expression increased in two compared with pretreatment values in rectal biopsy specimens (41). Our results in a larger patient group do not confirm these data. Although we recently postulated that sulindac could mediate its effect on intestinal adenoma formation by modifying p21 expression (19), the present study does not support such a mechanism.

In summary, in normal colorectal mucosa from HNPCC and FAP patients, sulindac had an inhibiting effect on β-catenin expression without affecting apoptotic indices and DR4, DR5, and p21 expression. Our data provide further support for inhibition of the Wnt signaling as a contributing mechanism of chemoprevention by sulindac. Whether this effect is universal or limited to patients genetically predisposed to colorectal cancer remains unclear.

References
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