Chapter 8

Sulindac inhibits β-catenin expression in normal appearing colon of hereditary nonpolyposis colorectal cancer and familial adenomatous polyposis patients.


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Abstract

Background: 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 (DR) pathway. Alternatively, mechanisms involving the APC-β-catenin-Wnt pathway have been suggested, possibly mediated by p21. The effects of sulindac were determined on apoptosis and expression of DR4 and DR5, β-catenin and p21 in normal appearing colorectal epithelium.

Methods: Biopsies were obtained before and after sulindac treatment during two chemoprevention studies. Patients (n = 18) with hereditary non-polyposis colorectal cancer (HNPCC) received 150 mg sulindac twice daily for 4 weeks in a placebo-controlled cross-over design. Patients (n = 6) with familial adenomatous polyposis (FAP) received 150 mg sulindac twice daily for 6 months. Apoptosis was assessed by M30 immunoreactivity and expression patterns of DR4, DR5, β-catenin and p21 were studied by immunohistochemistry.

Results: In HNPCC patients, apoptotic indices were similar following placebo and sulindac. Also in FAP patients, apoptotic indices were not different after sulindac compared to pre-treatment 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 HNPCC samples following sulindac compared to placebo (p < 0.001). Similar results were obtained in FAP samples (p < 0.01). p21 expression before and after sulindac treatment was similar, in both patient groups.

Conclusion: Sulindac inhibits β-catenin expression in normal colorectal epithelium from HNPCC and FAP patients without affecting apoptotic indices and DR4, DR5 and p21 expression.

Introduction

Colorectal cancer (CRC) is the second leading cause of cancer death in the western world. Familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) are well-defined conditions predisposing to CRC. Several studies have established the chemopreventive effects of nonsteroidal anti-inflammatory drugs (NSAIDs) as sulindac and celecoxib in patients with FAP, while studies in HNPCC patients are ongoing.

The precise mechanisms by which NSAIDs mediate their effects are incompletely understood, but likely involve induction of apoptosis. Apoptosis is controlled via two major pathways, one originating at the cell membrane, the extrinsic pathway, and one involving the mitochondria, the intrinsic pathway. Apoptotic pathways originating at the cell membrane involve death receptors like FAS, TNF-R1, DR3, DR4 and DR5, which are activated upon binding to their respective ligands. Recent reports reveal that sulindac mediates apoptosis through the mitochondrial pathway in colon cancer cells, involving caspase 9 and BAX. Sulindac-induced apoptosis has also been shown to involve DR5 as sulindac induced up-regulation of DR5 mRNA and protein levels, but not of DR4 in vitro.
The initial event in the neoplastic transformation of normal colon epithelium is assumed to be the activation of the Wnt-signalling pathway, caused by mutations in the APC or the β-catenin gene. This leads to cytoplasmic and subsequent nuclear accumulation of β-catenin. In the nucleus, β-catenin binds and activates the transcription factor TCF4. Finally, activated TCF4 activates a program presumed to be responsible for early adenoma formation. Several in vitro studies suggest that sulindac mediates its antineoplastic effect by inhibition of the Wnt-pathway. This is supported by studies in APC min mice and in adenomas of FAP patients. Recent reports reveal that sulindac affects Wnt-signalling by modifying expression of p21, a cyclin-dependent kinase inhibitor. To provide further insight into mechanisms involved in the chemopreventive action of sulindac, we studied the effects on apoptosis and expression of DR4, DR5, β-catenin and p21 in normal epithelium of FAP and HNPCC patients.

Materials and Methods

Patient selection.

Recently, a chemoprevention biomarker study was performed in proven or probable HNPCC patients at the University of Groningen Medical Centre. Proven patients were carriers of a mutation in one of the mismatch repair genes (hMLH1, hMSH2, hMSH6). Probable HNPCC patients had a family history meeting the revised Amsterdam criteria and a medical history of a 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 randomised double-blind placebo-controlled cross-over 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 Centre. After a wash out period of 4 weeks, patients crossed over to the alternative treatment for another 4 weeks. Full colonoscopy was performed at 4 and 12 weeks. Biopsies were taken of macroscopically normal mucosa with a standard 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 an NSAID in the three 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 hMLH2 (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. From 6 patients, tissue sections were available from normal appearing rectal mucosa before and after 6 months of treatment. These 6 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 deparaffinised 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. Staining procedures for M30, DR4 and DR5 were performed as previously described. For β-catenin staining (1:1000; clone 14, Transduction Laboratories, Lexington, KY, USA), antigen retrieval was carried out by microwave treatment for 8 min at 700 W in 0.01 M citrate buffer (pH 6.0). For p21 staining (1:150, clone WAF1, Oncogene Research), antigen retrieval was performed by heating slides three times for 5 min at 115 °C with 5 min cooling in between in maleate buffer in a preheated autoclave (Presto deluxe, Presto, Eclaire, WI, USA). After blocking of endogenous peroxidase with 0.3 % hydrogen peroxide for 30 min and incubation with avidin and biotin blocking solutions (Vector Laboratories, Burlingame, CA, USA), primary antibodies were applied for 1 h at room temperature. After washing with phosphate-buffered saline, slides were incubated with appropriate secondary and tertiary antibodies. Slides were counterstained with haematoxylin. As negative controls, slides were stained in absence of the primary antibody. As positive controls, sections of normal human liver (DR4, DR5) and colorectal cancer (β-catenin, p21, M30) were included. For each antibody, slides were stained in one batch.

**Evaluation of staining**

Slides were evaluated independently 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 index respectively). Only complete longitudinal crypts and at least 500 cells were counted. Intensity of DR4, DR5 and β-catenin staining was graded semi-quantitatively using a scale from 1 to 3 (1: weak staining; 2: moderate staining; 3: intense staining). For β-catenin, staining was separately 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 re-evaluated using a multi-headed microscope and the final grade was reached by consensus.

**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 chi-square tests. To determine differences between various colonic regions in HNPCC patients, Mann-Whitney tests for continuous variables and chi-square tests for discontinuous variables were conducted. Differences between proven and probable HNPCC patients were assessed using Mann-Whitney test for continuous variables and chi-square tests for discontinuous variables. Reported p-values are two-tailed and significance was assumed if p < 0.05. SPSS for Windows software (SPSS Inc, Chicago, IL) was utilised for all statistical analyses.
Effects of sulindac on normal colon in HNPCC and FAP

Results

To assess changes in apoptosis, DR4, DR5, β-catenin and p21 expression following placebo and sulindac, samples were analysed pair-wise, comparing staining results in biopsies from the same patient in the same colonic region. The analysis of sample pairs was hampered by limited availability of material. In case one of 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 analysed 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 analysed from FAP patients was 6 for all staining procedures.

Apoptosis

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

**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. \(\beta\)-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 \(\beta\)-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 \(\beta\)-catenin expression following sulindac (HNPCC and FAP)

Alterations in expression patterns of DR4, DR5 and \(\beta\)-catenin were analysed 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 2 sample pairs per patient were available.

Table 2 summarises changes in the distribution of staining intensities of DR4, DR5 and \(\beta\)-catenin in sample pairs from normal colon mucosa following sulindac, compared to placebo (HNPCC) and baseline values (FAP).

<table>
<thead>
<tr>
<th>Staining score</th>
<th>HNPCC</th>
<th>Placebo</th>
<th>Sulindac</th>
<th>FAP</th>
<th>Baseline</th>
<th>Sulindac</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n/n tested (%)</td>
<td>n/n tested</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DR4</td>
<td></td>
<td>5/64 (8%)</td>
<td>6/64 (9%)</td>
<td>0/6</td>
<td>1/6</td>
<td>1/6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>31/64 (48%)</td>
<td>32/64 (50%)</td>
<td>5/6</td>
<td>4/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>28/64 (44%)</td>
<td>26/64 (41%)</td>
<td>1/6</td>
<td>1/6</td>
<td></td>
</tr>
<tr>
<td>DR5</td>
<td></td>
<td>10/67 (15%)</td>
<td>9/67 (14%)</td>
<td>4/6</td>
<td>3/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>36/67 (54%)</td>
<td>39/67 (58%)</td>
<td>2/6</td>
<td>3/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>21/67 (31%)</td>
<td>19/67 (28%)</td>
<td>0/6</td>
<td>0/6</td>
<td></td>
</tr>
<tr>
<td>(\beta)-catenin</td>
<td></td>
<td>4/48 (8%)</td>
<td>9/48 (19%)</td>
<td>0/6</td>
<td>2/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>24/48 (50%)</td>
<td>33/48 (69%)</td>
<td>4/6</td>
<td>3/6</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>20/48 (42%)</td>
<td>6/48 (12%)</td>
<td>2/6</td>
<td>1/6</td>
<td></td>
</tr>
</tbody>
</table>

* Assessed as described in the Materials and Methods section
† number of samples varied in patient groups as a consequence of limited availability of slides
‡ distribution of staining intensities of \(\beta\)-catenin in HNPCC following placebo vs sulindac, p < 0.001
§ distribution of staining intensities of \(\beta\)-catenin in FAP at baseline vs sulindac, p < 0.01
Effects of sulindac on normal colon in HNPCC and FAP

In sample pairs, the intensity scores of DR4 staining were not consistently different following sulindac compared to placebo in HNPCC: higher in 26/64 pairs, lower in 27/64 pairs and unchanged in 11/64 pairs (not significant). For DR5, similar results were obtained: higher in 19/67, lower in 20/67 and unchanged in 28/67 pairs (not significant). The intensity scores of membranous β-catenin staining following sulindac compared to placebo was higher in 7/48, lower in 26/48 and unchanged in 15/48 pairs (p < 0.05). In paired FAP samples, DR4 and DR5 staining intensities were similar between baseline and sulindac in all 6 samples. For membranous β-catenin, staining intensities were lower following sulindac in 3/6 and unchanged in 3/6 FAP pairs (not significant). In cases with lower staining intensity of membranous β-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 β-catenin, cumulative intensity scores were significantly lower following sulindac compared to placebo (p < 0.01, figure 1).

**p21**

Mean percentages of p21 positive cells following placebo and sulindac are shown in table 1 (right panel). 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 to placebo (HNPCC) and baseline (FAP) values.
Chapter 8

Discussion

The efficacy of chemopreventive agents in the colorectum is routinely assessed by measuring one or more endpoints: biomarker modulation in the at-risk mucosa, adenoma regression, adenoma suppression or adenoma prevention. Our biomarker modulation study evaluated changes in apoptosis and expression of DR4, DR5, β-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 NSAIDs. 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 to 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 NSAIDs in the setting of chemoprevention of CRC. An important mechanism behind the chemopreventive effect of sulindac appears to be induction of apoptosis. Sulindac is a prodrug that is converted into sulindac sulfide and then sulindac sulfone by colonic bacteria. In vitro, both metabolites induce apoptosis in colon cancer cells, including mismatch repair deficient cells. In APC^Min^ mice, a mouse model of FAP, sulindac had an anti-tumour effect and was associated with induction of apoptosis. Also in a mismatch repair deficient APC^Min^ mouse model, carrying genetic features of both FAP and HNPCC, sulindac inhibited intestinal adenoma development. 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. 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 FAP patients, no changes in apoptosis were seen upon sulindac treatment. In accordance with these data, sulindac did not have a preventive effect on the development of adenomas in these phenotypically unaffected patients. There is no data on efficacy of chemoprevention in HNPCC patients, although studies are ongoing. Taken together, the chemopreventive action of sulindac in FAP patients seems to be 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 NSAIDs. In vitro, NSAIDs including sulindac, prevented nuclear accumulation of β-catenin. Oncogenic activation of the Wnt-signalling pathway resulting in nuclear translocation of β-catenin is considered critical for the initiation in intestinal epithelial neoplastic transformation. A recent report reveals that adenomas from FAP patients showed less nuclear β-catenin staining after sulindac treatment. Similar results were obtained in APC^Min^ mice, in normal intestinal mucosa as well as in adenomas. 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 is unknown.

Finally, we assessed whether changes in β-catenin expression were associated with alterations in p21 expression. Recent data indicated that active Wnt-signalling decreases p21 concentrations preventing cells from entering G1 arrest or differentiation, thereby allowing cells to proliferate. In a previous study of three patients treated with sulindac, p21 expression increased in two compared to pre-treatment values in rectal biopsy specimens. 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, 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, DR4, DR5 and p21 expression. Our data provide further support for inhibition of the Wnt-signalling as a contributing mechanism of chemoprevention by sulindac. Whether this effect is universal or limited to patients genetically predisposed to colorectal cancer is unclear.
Chapter 8

References


