Gastrointestinal polyposis syndromes
Keller, J.J.

Citation for published version (APA):

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Download date: 17 Oct 2018
Chapter 3

Rectal epithelial apoptosis in familial adenomatous polyposis patients treated with sulindac.


Department of Pathology, Academic Medical Center, Amsterdam
Oncology Centre, Johns Hopkins University School of Medicine, Baltimore, MD, USA
Department of Pathology, Johns Hopkins University Hospital, Baltimore, MD, USA
Department of Medicine, Johns Hopkins University Hospital, Baltimore, MD, USA

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Abstract

Background—Sulindac regresses colorectal adenomas in patients with familial adenomatous polyposis (FAP), although the mechanism of polyph regression is unclear. Aims—To determine whether differences occur in alteration of rectal epithelial apoptotic index and expression of apoptosis related proteins in FAP patients treated with sulindac compared with placebo.

Patients—Twenty one FAP patients; 12 had not undergone colectomy.

Methods—Patients with FAP were treated with sulindac 150 mg orally twice a day for three months (n=10) or placebo (n=11). Colorectal polyp number was determined and biopsies of the normal rectal mucosa were performed before and after three months of treatment. Response to treatment and alteration of the apoptotic ratio (Index in base of crypt divided by index in surface epithelium) were evaluated. Bcl-2, bax, p21/WAF-1, and p53 proteins were assessed semiquantitatively by immunohistochemistry.

Results—Significant decreases in polyp number and in the apoptotic ratio were seen in patients treated with sulindac compared with controls. The mean percentage change in polyp number from baseline was -46% in the sulindac group and +13% in the placebo group (p=0.005). Mean percentage change in the apoptotic ratio was -8% and +25% in the sulindac and placebo treated patients, respectively (p=0.004). No differences in expression or compartmentalisation of apoptosis related proteins were noted between treatment groups.

Conclusions—Sulindac regression of colorectal adenomas is accompanied by alteration of the rectal epithelial apoptotic ratio with relative increase in apoptosis in surface cells compared with the deeper crypt. The utility of the apoptotic ratio as an intermediate biomarker for colorectal tumorigenesis deserves further study.

Keywords: apoptosis; familial adenomatous polyposis; sulindac; Intermediate biomarker; tumorigenesis

Multiple lines of investigation support the concept that non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin can prevent colorectal cancer. Specifically, NSAIDs inhibit cell growth in cell culture,

Further discusses the utility of the apoptotic ratio as an intermediate biomarker for colorectal tumorigenesis, highlighting the importance of these findings in the context of colorectal cancer prevention.
Therefore, we investigated the effects on apoptosis in individual crypt compartments of grossly normal appearing flat rectal epithelium of FAP patients treated with sulindac compared with placebo. The expression and compartmentalisation of apoptosis related protein expression were also analysed in these two patient groups.

Methods
Subjects
Patients with FAP were enrolled in a randomised, double blind, placebo controlled trial to determine the effect of sulindac on colorectal adenomas, as reported previously. Twenty one patients from the larger initial study population (12 who had not undergone colectomy) with adequate colorectal mucosal samples at time 0 and three months were analysed in this study. Informed consent was obtained from all patients, and the protocol was approved by The Johns Hopkins University Joint Committee on Clinical Investigation (institutional review board). Ten patients (three men, seven women; mean age 26.5 (SD 10.1) years, range 13–45) received 150 mg sulindac by mouth twice a day for three months. Eleven patients (six men, five women; mean age 25.7 (SD 8.7) years, range 16–51) took identical placebo tablets for three months. Compliance with medication was assessed by pill count and was universally greater than 85%. At time 0 and 3 months, rectal polypl number was assessed by flexible sigmoidoscopy using an Olympus flexible video sigmoidoscope. At time 0 the colorectal mucosa was tattooed with sterile India ink about 20 cm from the anal verge. The endoscopist counted total polypl number in the entire circumference of colorectum from the tattoo mark to anal verge and recorded the examination on videotape. There were no significant differences in the demographic and clinical characteristics between treatment groups.

All patients were prepared for each endoscopic procedure with a clear liquid diet and oral cathartic solution. Enemas which could influence mucosal biochemistry were not given. In each patient, during flexible sigmoidoscopy, six rectal mucosal biopsy specimens were taken from flat mucosa 10–12 cm from the anal verge to minimise potential differences which might occur from specimens taken at different colorectal sites. Rectal mucosal specimens were snap frozen in liquid nitrogen or placed in formalin or methanol, and embedded in paraffin wax for histopathological examination.

Detection of Apoptosis
Apoptotic cell death was assessed by light microscopy in a blinded fashion, with examination of coded haematoxylin and eosin stained slides from biopsy specimens of the grossly normal colorectal mucosa by two observers (GJAO, JJK) as utilised previously. Representative areas of the sections were selected at low power and then studied at 40× magnification. Cells were recognised as apoptotic bodies according to strict morphological criteria: cell shrinkage with retracted pink to orange cytoplasm, chromatin condensation and nuclear fragmentation, and separation of cells by a halo from adjacent entecytes. Only isolated apoptotic bodies were counted. A prominent reaction was absent. Intersitial lymphocytes were easily distinguished by their morphology and larger size. When a distinction could not be made between a lymphocyte and an apoptotic body, it was not counted.

Apoptotic index (AI) at the base of the crypt was assessed from the lowermost cell up to 20 cell positions along each side of the crypt ("proliferative compartment"). The number of apoptotic cells was counted and divided by the total number of epithelial cells in the proliferative compartment. The AI was determined for all longitudinal crypt sections in which the lumen of the crypt was completely visible. Only crypts in which extension of the crypt to the underlying muscularis mucosae was visible were included, ensuring only cells in the proliferative compartment of the crypt were counted. Crypts near a lymphoid aggregate, an inflammatory infiltrate, or nearby adenomatous epithelium were excluded.

The effect of sulindac on compartmentalisation of apoptosis was evaluated by analysing the apoptotic ratio (AI of crypt base divided by AI of surface).

Immunohistochemistry for Apoptotic Related Proteins
Immunohistochemistry for p21/WAF1, bcl-2, bax, and p53 protein expression and compartmentalisation was performed on the same formalin fixed paraffin wax embedded specimens of grossly normal colorectal mucosa as used for apoptotic counting. Immunohistochemistry was performed as described previously using citrate buffer for antigen enhancement and with final detection through standard avidin–biotin staining methods. The monoclonal antibodies DO7 (Dakopatts, Glostrup, Denmark) at a dilution of 1/200, Ab-1 (Onco gene Science, Cambridge, Massachusetts) at a dilution of 1/50, and Oncoprotein 124 (Dakopatts, Glostrup, Denmark) at a dilution of 1/50 were used for the detection of mutated p53, expression of p21/WAF1, and expression of bcl-2, respectively. Biotinylated rabbit antimouse (Dakopatts, Glostrup, Denmark) was used as secondary antibody. The protein bax was detected using the polyclonal rabbit IgG p19 (Santa Cruz Biotechnology Inc.) at a dilution of 1/100 and with a biotinylated swine antirabbit (Dakopatts, Glostrup, Denmark) as secondary antibody. Primary antibodies were replaced with phosphate buffered saline (PBS).
in negative control slides. Positive controls used were normal colon (p21/WAF-1), lymphoid infiltrates in the studied colorectal biopsy specimens (bcl-2), Paneth cells in small intestine (bax), and a known p53 positive colorectal carcinoma.

p21/WAF-1, bcl-2, bax, and p53 were evaluated in colorectal mucosa as described elsewhere. Coded slides were scored by two observers (GJAO, JJK) in a blinded fashion using light microscopy at 25× magnification.

STATISTICAL ANALYSIS
The major statistical endpoint evaluated in this study was the effect of sulindac treatment on...
Apoptosis. Apoptotic bodies in crypts in the proliferative compartment and within cells of the surface epithelium were studied. Data were collected for each patient at baseline and three months after receiving either sulindac or placebo treatment. Apoptotic indexes (number of apoptotic bodies divided by the total number of counted cells) were calculated for base of crypts and for surface epithelium. The apoptotic ratio (AR) was the ratio of these two numbers.

Statistical comparisons of changes in polyp number, apoptotic ratio, and protein expression were done using the non-parametric Wilcoxon rank sum test. To evaluate interaction effects (whether the sulindac effect differed in subjects with intact colons or rectal stumps), changes in these measures were treated as continuous variables in standard regression models. Because some of the pretreatment apoptotic ratios were zero, regression modelling of the relative AR change required a transformation of the apoptotic ratio, adding 1 to both the pretreatment and post-treatment ratios: [(post-treatment AR + 1) - (pretreatment AR + 1)]. All statistical computations were performed using the SAS (Statistical Analysis System), and all p values reported are two sided.

Results

Clinical Effect of Sulindac

The mean percentage change in polyp number from baseline was significantly decreased in the sulindac group (−46%) compared with the placebo group (+13%; p=0.005); change in polyp number (SD) was −11.5 (16.5), range −58 to 9 in the sulindac group, and 0.09 (16.6), range −33.0 to 29.0 in the placebo group. Sample size was too small to make reliable conclusions concerning differences in effect of sulindac on patients with intact colons compared with those with retained rectums.

Apoptosis

The mean number of crypts counted in each patient was 12.7 (range 4–32). The total number of cells counted in the sulindac group was 14,283 pretreatment and 12,010 post-treatment. In the placebo group, 13,438 and 12,539 were counted, respectively.

A significant decrease in AR (AI base/AI surface) was noted in the sulindac group following treatment at three months (table 1, fig 2). The mean percentage change in AR was −8% in the sulindac group and +25% in the patients on placebo (p=0.004); change in apoptotic ratio was −0.13 (0.29), range −0.58 to 0.48 in the sulindac group, and 0.29 (0.19), range −0.02 to 0.61 in the placebo group. In the sulindac treated patients, change in AR was due to an increase of apoptosis at the surface and a decrease in the lower part of the crypt (fig 3).

Apoptosis Related Protein Expression

Table 2 and fig 1 summarise p21/WAF1, bcl-2, and p53 protein expression in FAP patients in the sulindac and placebo groups pretreatment and post-treatment. p21/WAF1 was expressed in the nuclei at the luminal surface and the upper third of the crypts (fig 1B). No expression in the proliferative compartment was noted. Cytoplasmic bcl-2 staining was confined to cells in the crypt base (fig 1C). Only sporadic, faint staining of the surface epithelium occurred. Cytoplasmic bax staining was strongest at the luminal surface as...
The findings in the present study are consistent with the observations of Mahmoud et al in the MIN mouse model of familial adenomatous polyposis.6 Histologically normal MIN intestinal epithelium exhibits elevated B catenin expression associated with aberrant proliferation and apoptosis and a decreased rate of enterocyte crypt-villus migration. Tumour preventing doses of sulindac sulphide normalised enterocyte proliferation and apoptosis and restored a normal enterocyte migration pattern. The reason for the lack of normalisation of proliferation in some human studies remains unclear.

In normal colonic crypts, epithelial cell growth and function depend on a finely tuned homoeostasis of cell proliferation, migration, differentiation, and apoptosis. The APC protein seems to play a crucial role in this process through interaction with B catenin. APC controls B catenin by binding and phosphorylation, resulting in breakdown of the B catenin protein.46 B catenin is a component of the adherens junction, a cadherin associated transmembrane complex mediating cell adhesion and a possible pathway through which the APC protein modulates cell migration.47 Moreover, B catenin binds to the DNA binding proteins of the Wnt signalling pathway, regulating cell proliferation and apoptosis and APC inhibits B catenin/Tcf mediated transcription.48-50 Also, when only one allele is lost through germline mutation, the above functions of APC seem to be compromised already, both in humans and in MIN mice.51

Although the precise mechanism is unknown, the functions of the APC protein appear restored by sulindac sulphide.51 Induction of apoptosis correlates with cyclooxygenase 2 inhibition by sulindac, with subsequent increase in lipid compounds including ceramide which induces apoptosis.52 The apoptotic ratio in the colorectal mucosa proved useful in distinguishing patients on and off sulindac treatment. Of particular interest is a 24 year old woman treated with sulindac who developed colorectal cancer with concomitant complete regression of adenomas. Similar to other patients with adenoma resolution, biomarkers including mucosal prostaglandin concentrations dropped dramatically with sulindac therapy.53 But in contrast, the apoptotic ratio in this patient's colorectal mucosa revealed no change from presulindac treatment, a finding strikingly different from the others with adenoma regression (fig 2). Consequently, the value of the apoptotic ratio as an intermediate biomarker deserves further investigation.

The present study failed to find that the changes in the apoptotic ratio were accompanied by differences in expression or compartmentalisation of WAF-1/p21, bcl-2, bax, or p53 proteins between sulindac and placebo treatment groups. Similarly, other investigators utilising in vitro models have reported that sulindac-induced apoptosis is independent of bcl-2 expression and the integrity of the p53 tumour suppressor pathway.54 However, Goldberg et al found increased levels of p21 and reduced levels of mutant p53 in the HT29

Table 2 Median (range) protein expression and localisation in patients treated with sulindac and placebo

<table>
<thead>
<tr>
<th>Protein</th>
<th>Sulindac</th>
<th>Placebo</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>bcl-2</td>
<td>Base</td>
<td>3 (2-3)</td>
</tr>
<tr>
<td></td>
<td>Surface</td>
<td>1 (1)</td>
</tr>
<tr>
<td>bax</td>
<td>Base</td>
<td>2 (2)</td>
</tr>
<tr>
<td></td>
<td>Surface</td>
<td>3 (3-4)</td>
</tr>
<tr>
<td>p21</td>
<td>Base</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>Surface</td>
<td>3.5 (2-4)</td>
</tr>
<tr>
<td>p53</td>
<td>Base</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>Surface</td>
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p21 was only expressed in the upper third of the crypt and at the luminal surface; no p53 expression was seen. No statistical differences were noted by Wilcoxon paired rank sum test.

compared with the base of the crypts (fig 1C).

There were no differences in expression of WAF-1/p21, bcl-2, or bax before or after treatment with sulindac. The p53 gene product was not over expressed in normal colorectal mucosa of any patient before or after treatment with sulindac.

Discussion

Results of numerous investigations have concluded that NSAIDs are chemoprotective agents against colorectal tumorigenesis. The mechanism of chemoprevention of NSAIDs remains unclear, but which after expression macro evidence suggests an effect of these drugs on apoptotic pathways in colorectal epithelial cell kinetics. Previously, we reported that sulindac produced colorectal adenoma regression in patients with familial adenomatous polyposis and affected epithelial cell apoptosis but not proliferation in their colorectal mucosa.53 The lack of effect of sulindac on colorectal cell proliferation in FAP patients has also been noted by others.54 In addition, both animal and cell culture experiments have confirmed the chemopreventive property of sulindac and the ability of this agent to induce apoptosis.

In normal colorectal mucosa, proliferation and apoptosis occur in well defined zones. Proliferating cells are restricted to the lower two thirds of the crypt, whereas apoptosis occurs principally at the luminal surface between crypts.55 Importantly, in FAP patients alterations in cell kinetics in normal appearing flat colorectal mucosa are noted with upward shift of the proliferative compartment and decreased surface apoptosis,56 57 the earliest changes noted in adenoma formation. Therefore, in the present study, the compartmentalisation of apoptosis was evaluated in patients with familial adenomatous polyposis before and after treatment with sulindac in comparison to placebo. In patients who had sulindac induced regression of colorectal adenomas, alteration of the colorectal mucosal apoptotic ratio with relative increase in apoptosis in surface epithelium compared with crypt base was noted. This shift towards apoptosis in the surface epithelium is consistent with normal epithelial cell kinetics and discordant with the pattern noted in the adenomas-carcinoma sequence in which apoptosis is reduced at the surface epithelium of adenomas and occurs more frequently at the base of the adenomatous crypt.58 59
colon cancer cell line after exposure to sulindac. Yet, these effects appeared to occur independent of the ability of sulindac to induce apoptosis and were not associated with cell differentiation. A recent study showed influence of low dose sulindac on expression of the p53 tumor suppressor gene and bcl-2, but these investigators utilized different methodology. Although no differences in expression or compartmentalisation of specific gene expression was noted in our study, smaller differences may have been missed by the immunohistochemical methodology utilised, the small number of patients studied, or the small number of categories evaluated. In summary, this study provides evidence in humans that sulindac interferes with the regulation of rectal epithelial cell kinetics by altering the apoptotic ratio in the rectal epithelium. Additionally, the apoptotic ratio may be a useful intermediate biomarker in the study of colorectal tumorigenesis.

This work was supported in part by The Clayton Foundation, The National Institutes of Health (1 R01 CA70000), and NIH grant CA58301, CA634, and CA62091. GJA Offiheras is supported by the Queen Wilhelmina Foundation, Dutch Cancer Foundation for visiting professorship at The Johns Hopkins University School of Medicine. J. Koles was also supported by the Queen Wilhelmina Foundation for a research elective at The Johns Hopkins Medical Institutions. The authors thank Ms Linda M. Welch for secretarial support; Drs Annoncio Caron-Jansen for critically reviewing the manuscript and for providing helpful suggestions; Drs Marc Shoham and Dehoux for donating sulindac and placebo.