Part I: Chemoprevention of the Adenoma-Carcinoma Sequence

Chapter 6

NSAIDs target nuclear β-catenin accumulation and Wnt signaling in adenomas of patients with familial adenomatous polyposis and in colorectal cancer cell lines

Elles M.J. Boon, Josbert J. Keller, Thera A.M. Wormhout, G. Johan A. Offerhaus, Francis M. Giardiello, Ronald van der Neut, Steven Pals

Department of Pathology, Academic Medical Center, Amsterdam, The Netherlands
Department of Medicine, Johns Hopkins University Hospital, Baltimore, MD, USA

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NSAIDs target nuclear β-catenin accumulation and Wnt signaling in adenomas of patients with familial adenomatous polyposis and in human colorectal cancer cell lines

Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) have chemopreventive potential against colorectal carcinomas (CRC). Inhibition of COX-2 activity underlies part of this effect, although COX-2-independent mechanisms may also exist. NSAIDs appear to inhibit the initial stages of the adenoma-carcinoma sequence, suggesting a link to the APC/β-catenin/TCF-pathway (Wnt-signaling pathway). Therefore, the effect of the NSAIDs sulindac and indomethacin on nuclear (non-phosphorylated) β-catenin and β-catenin/TCF mediated transcription was investigated. Nuclear β-catenin expression was assessed in pretreatment colorectal adenomas and in adenomas after treatment with sulindac from 7 patients with familial adenomatous polyposis (FAP). Also, the effect of indomethacin and sulindac on β-catenin/TCF mediated transcription was studied. Adenomas of FAP patients collected after treatment with sulindac for up to 6 months showed less nuclear β-catenin expression compared to pretreatment adenomas of the same patients. Both indomethacin and sulindac abrogated β-catenin/TCF mediated transcription in the CRC cell lines DLD1 and SW480, and decreased the levels of non-phosphorylated β-catenin. As a result, the protein levels of the positively regulated TCF-targets Met and Cyclin D1 were downregulated, while the protein level of the negatively regulated TCF-target CD68 was upregulated after indomethacin or sulindac treatment. This study provides in vivo and in vitro evidence that nuclear β-catenin localization and β-catenin/TCF regulated transcription of target genes can be inhibited by NSAIDs. Inhibition of Wnt-signaling provides an explanation for the COX-2-independent mechanism of chemoprevention by NSAIDs.

Introduction

Epidemiological data, rodent studies and in vitro experiments have demonstrated that non-steroidal anti-inflammatory drugs (NSAIDs) have anti-colorectal cancer (CRC) activity. Also, in patients with familial adenomatous polyposis (FAP), an autosomal dominantly inherited disorder characterized by the development of numerous colorectal adenomas at a young age, the NSAIDs sulindac and indomethacin can cause regression of adenomas. The chemopreventive effect of NSAIDs appears mediated by induction of apoptosis and cell cycle arrest. However, the molecular mechanisms underlying these biological effects are not completely understood. NSAIDs inhibit the enzymatic activity of cyclooxygenase (COX) 1 and 2, enzymes that convert arachidonic acid into prostaglandins. However, COX independent mechanisms may also play a role, since NSAIDs inhibit the growth of colon cancer cell lines lacking COX-2 expression.

Oncogenic activation of the Wnt signaling pathway by mutations in APC or β-catenin, which results in accumulation and nuclear translocation of β-catenin and in β-catenin/TCF4 regulated transcription of TCF target genes, is mandatory for the initial neoplastic transformation of intestinal epithelium. Previous studies have shown an effect of NSAIDs on the expression
and localization of β-catenin, suggesting nuclear β-catenin as an alternative target for the chemopreventive effect of NSAIDs. NSAIDs were shown to prevent the nuclear accumulation of β-catenin in chemically induced colon tumors in rats 17 and in human colorectal cancer cell lines 14,18. In addition, indomethacin and aspirin can downregulate the expression of the TCF target gene cyclin D1 in CRC cell lines 18,19. Together, these data suggest that NSAIDs may exert an anti-neoplastic effect by inhibiting the Wnt-signaling pathway. Previously, we reported low levels of nuclear β-catenin in sulindac resistant adenomas 20. This could reflect a downregulation of nuclear β-catenin by sulindac or represent an intrinsic feature of resistant adenomas. In the present study, we therefore compared nuclear accumulation of β-catenin in adenomas from FAP patients before and after treatment with sulindac for up to 6 months 6. In addition, we studied the effects of indomethacin and sulindac on Wnt-signaling in human CRC cell lines.

Methods

Patients and adenoma specimens

The study population consisted of 7 FAP patients who were treated with sulindac 150 mg p.o. twice a day, as described previously 6,21,22. All patients had adenomas at the initiation of treatment (baseline) and showed adenoma regression after 6 months treatment with sulindac. Patients were selected because both adenomas collected at baseline (n=17) and after the first 6 months of treatment with sulindac (n=17) were available for study.

Immunohistochemistry for β-catenin

Immunohistochemistry was performed on 5 μm sections of formalin-fixed, paraffin-embedded samples as described previous 23. For antigen retrieval, the slides were boiled for 10 min. in citrate buffer followed by an overnight incubation at 4 °C with a primary monoclonal antibody against β-catenin, clone 14 (Transduction Laboratories, Lexington, KY). The staining pattern in adjacent normal mucosa was used as a marker for specificity. Immunostained slides were scored semiquantitatively using a scale from 0 to 3 (0: no expression; 1: ≤5% positive nuclei; 2: 5-25% positive nuclei; 3: ≥25% positive nuclei). Also, membranous staining was scored separately as normal or decreased. Slides were assessed in a coded fashion by two independent observers (JJK and GJAO) and discrepancies were solved by consensus. Comparisons of staining patterns between adenomas collected at baseline and during treatment with sulindac were made using the non-parametric Mann-Whitney test and Fisher’s Exact test. A P<0.05 was considered statistically significant; P-values were two-sided.

Cell culture and TCF reporter analysis

The human CRC cell lines DLD1 and SW480 were grown in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin (all from Life Technologies, Paisley, UK). CRC cells were transiently transfected with either 5 μg pTOPflash or pFOPflash reporter plasmids (Upstate Biotechnology, Lake Placid, NY) using lipofectamine (Invitrogen, Paisley, UK). To correct for differences in transfection efficiency, cells were cotransfected with a reporter plasmid containing a GFP gene. All experiments were
performed in triplicate. Cells were grown with or without addition of indomethacin (600 μM) or sulindac (100 μM) for 24 or 48 hours. The concentration ranges of indomethacin and sulindac in this study were equivalent to that used by others. Indomethacin and sulindac were prepared as stock solution in dimethyl sulfoxide (DMSO). Control cultures contained DMSO at an equivalent dilution. After stimulation of the cells with indomethacin or sulindac, cells were lysed in luciferase reporter lysis buffer (Promega, Madison, NY) and monitored for luciferase activity using luciferase assay substrate buffer. Light units were recorded using a luminometer. GFP expression of these cells was analyzed by microscopy to correct for differences in transfection efficiency.

**Western Blot analysis**

The CRC cells DLD1 and SW480 were grown with or without 600 μM indomethacin or 100 μM sulindac for 24 or 48 hours. In addition, cells were grown with different concentrations of indomethacin or sulindac for 24 hours. Protein extracts were prepared by resuspending the cells into lysis buffer (10 mM Tris pH 8.0; 15 mM NaCl; 1% NP40, 10% glycerol; 0.4 mg/ml sodium orthovanadate). Protein extracts were separated by SDS-PAGE and blotted onto immobilon-P transfer membranes (Millipore corp., Bedford, USA) by tank blotting. Membranes were blocked in Tris buffered saline (100 mM Tris-Cl pH 7.5; 150 mM NaCl) containing 0.1 % Tween (Sigma, St Louis, MO) and 5 % non-fat dry milk, probed with monoclonal antibodies; 8E4 (against non-phosphorylated β-catenin) (Alexis Biochemicals, San Diego, CA), KP1 (against CD68) (Research Diagnostics Inc, Flanders, NJ) and AC-15 (against β-actin) (Sigma), respectively polyclonal antibodies: H-102 (against β-catenin), C12 (against Met), M-20 (against cyclin D1) (all Santa Cruz Biotechnology, Santa Cruz, CA). Proteins were detected with horseradish peroxidase conjugated secondary antibodies (Dakopatts, Glostrup, Denmark) in a standard Western Blotting protocol (ECL Western Blotting, Amersham Pharmacia Biotech Inc., Aylesbury, UK). Blots were analyzed by densitometry. Intensities were quantified using NIH Image software (NIH, USA). All signals were normalized for loading by comparison with the appropriate β-actin signal.

**Figure 1. β-Catenin expression in adenomas of FAP patients:** Immunostaining of adenomas from FAP patients before (A) and after treatment with sulindac (B). After sulindac treatment, nuclear β-catenin staining (arrow) is diminished (arrowhead).
Results

\(\beta\)-Catenin expression in adenomas before and after sulindac treatment

Thirty-four adenomas from FAP patients removed before (n=17) and after (n=17) treatment with sulindac for up to 6 months (median duration of treatment per adenoma: 4 months) were evaluated. All adenomas were tubular or tubulovillous lesions, <1 cm, with mild to moderate dysplasia. No morphological differences were noted between adenomas removed before and after treatment. Nuclear accumulation of \(\beta\)-catenin was assessed semiquantitatively in immunostained slides. There was nuclear accumulation of \(\beta\)-catenin in baseline adenomas compared to adenomas collected after treatment with sulindac (Figure 1). Immunostained slides were scored semiquantitatively as described in the Materials and Methods section and summarized in Table 1. Membranous \(\beta\)-catenin was decreased in all baseline adenomas, and in 14 out of 17 adenomas removed after sulindac (P>0.05).

<table>
<thead>
<tr>
<th># of adenomas</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
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<tr>
<td>before treatment</td>
<td>0</td>
<td>9</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>after treatment</td>
<td>6</td>
<td>9</td>
<td>2</td>
<td>0</td>
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NSAIDs target \(\beta\)-catenin/TCF activated transcription

The effect of NSAIDs on Wnt-activation was analyzed in the human colorectal cancer cell lines DLD1 and SW480. These cell lines contain a constitutively activated Wnt signaling pathway caused by mutations in the APC gene, and are devoid of COX-2 expression (and confirmed by western blotting, data not shown). Inhibition of \(\beta\)-catenin/TCF transcriptional activation by indomethacin or sulindac was monitored by transfecting a TCF reporter (pTOPflash) or, as control, a construct containing scrambled TCF binding sites (pFOPflash) in both CRC cell lines. Differences in transfection efficiency were corrected for by cotransfecting the cells with a GFP-encoding reporter plasmid. Following indomethacin (600 \(\mu\)M) or sulindac (100 \(\mu\)M) treatment for 24 or 48 hrs, \(\beta\)-catenin/TCF activated TOPflash activity was abrogated in both DLD1 and SW480 cells (Figure 2). FOPflash activity remained low in both cell lines. This indicated that indomethacin and sulindac inhibited the Wnt signaling pathway.

To further support this conclusion, we determined the effect on (i) the phosphorylation status of \(\beta\)-catenin and on (ii) the expression of TCF target genes. In the absence of active Wnt-signaling, \(\beta\)-catenin is phosphorylated by Glycogen synthase kinase3\(\beta\) (GSK3\(\beta\)) and targeted for degradation by the ubiquitin-proteasomal pathway. Mutations in the APC gene, which are present in both DLD1 and SW480 cells, result in reduced phosphorylation of \(\beta\)-catenin and nuclear accumulation of non-phosphorylated \(\beta\)-catenin. Following indomethacin (Figure 3 and 4) and sulindac (data not shown) treatment, a time- and dose-dependent decrease in non-phosphorylated
β-catenin expression in both cell lines was noted. Furthermore, the expression levels of the TCF target genes Met \(^2^4\) and Cyclin D1 \(^2^5\) were downregulated in a time- and dose-dependent fashion. By contrast, CD68, a TCF target gene normally downregulated by TCF directed transcription \(^2^6\), was upregulated in response to treatment.

Discussion

In addition to COX-2, a number of alternative targets have been implicated in the chemopreventive action of NSAIDs, including Bcl-2, NF-κB, NAG-1 and PPAR\(^6\) \(^2^7\)-\(^3^0\), suggesting that various distinct molecular pathways may play a role in the anti-tumor effects of these drugs. The present study provides in vivo and in vitro evidence that the β-catenin/TCF-4 signaling pathway is a target of NSAIDs.

We observed that nuclear β-catenin accumulation in adenomas of FAP patients treated with sulindac is decreased in comparison to pretreatment adenomas of the same patients. A similar decrease in nuclear β-catenin has been reported in rodent intestinal tumors \(^1^7\). Previously, we found less nuclear β-catenin in sulindac-resistant adenomas compared to baseline adenomas \(^2^0\). Those sulindac resistant adenomas were collected during treatment with sulindac for up to 4 years, from selected sulindac resistant patients. Therefore, our previous study could not distinguish whether decreased nuclear β-catenin was caused by sulindac, or reflected intrinsic features of sulindac resistant adenomas. The present study compared adenomas from FAP patients
removed before and after treatment with sulindac for up to 6 months. At that timepoint, the maximum efficacy of sulindac on number and size of adenomas was noted\(^6\), and in general resistance started to develop afterwards. Thus, it is assumed that the observed decrease of nuclear \(\beta\)-catenin in the present investigation is mostly accounted for by the sulindac treatment, instead of being related to resistance. This corresponds to data showing decreased nuclear and cytoplasmic \(\beta\)-catenin in adenomas of Apc\(^{Min}\) mice upon treatment with sulindac for several days\(^7\).

Figure 3. Kinetics of the effect of indomethacin on TCF-regulated target genes. DLD1 (A) and SW480 cells (B) were treated with 600 \(\mu\)M indomethacin for 24 or 48 hrs. Protein lysates were analysed by Western blot using antibodies against total \(\beta\)-catenin, non-phosphorylated \(\beta\)-catenin, Met, Cyclin D1 and CD68. Blots were analyzed by densitometry and each signal was normalized for loading by comparison with the appropriate \(\beta\)-actin signal (C). Data shown are mean values ± SD from 3 independent experiments.

<table>
<thead>
<tr>
<th>DLD1</th>
<th>SW480</th>
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<tr>
<td>Indomethacin</td>
<td>0 24 48 hrs</td>
</tr>
<tr>
<td>(\beta)-Catenin</td>
<td></td>
</tr>
<tr>
<td>Nonphospho-(\beta)-catenin</td>
<td></td>
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<tr>
<td>Met</td>
<td></td>
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<td>Cyclin D1</td>
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<td>(\beta)-Actin</td>
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\(\beta\)-catenin protein expression

Nonphospho \(\beta\)-catenin protein expression

Met protein expression

Cyclin D1 protein expression

CD68 protein expression
The observation that NSAIDs decrease nuclear β-catenin in adenomas in vivo is of interest, suggesting a direct link between the tumor suppressive effects of NSAIDs and the key defect of colorectal cancer, i.e. deregulated Wnt-signaling. Mutations involving components of the Wnt signaling cascade, specifically in APC or β-catenin, are essential for the initiation of colorectal cancer. In the normal intestinal epithelium, these molecules are part of a multiprotein-complex in which β-catenin is phosphorylated by GSK-3β and targeted for degradation by the ubiquitin-proteasomal pathway. Mutations in APC or β-catenin lead to dissociation of the complex, causing accumulation of non-phosphorylated β-catenin, which translocates to the nucleus and acts as a transcriptional co-activator of TCF transcription factors. Our observation that sulindac treatment diminishes the nuclear accumulation of the transcriptional co-activator β-catenin in adenomas of FAP patients in vivo suggests that NSAIDs exert tumor suppressive effects by interfering with TCF-mediated transcription. In line with these in vivo data, we observed that NSAID treatment of the CRC cell lines DLD1 and SW480 suppresses TCF reporter activity. Furthermore, NSAID treatment decreased the expression of the TCF target genes Met [24] and Cyclin-D1 in a time- and dose-dependent fashion. These suppressive effects were not due to generalized transcriptional repression, since expression of CD68, a negatively regulated TCF target gene, was upregulated.

NSAID treatment only led to a moderate decrease in total β-catenin levels. A similar finding was reported by Smith and co-workers [14]. By contrast, Dihlmann et al. reported no appreciable effect on β-catenin levels, although a change in TCF-mediated transcription was found [19]. We have no explanation for this discrepancy. However, the validity of our finding is supported by the observation that the decrease in non-phosphorylated β-catenin (which represents the transcriptionally active portion of β-catenin) was more pronounced than the decrease of total
β-catenin, suggesting a selective effect of NSAIDs on the pool of β-catenin that is involved in Wnt-signaling. The CRC cells used in our study carry an APC mutation, preventing formation of the APC/β-catenin multiprotein complex. Therefore, it seems unlikely that the NSAID-induced decrease in non-phosphorylated β-catenin is regulated by GSK-3β activity. Other pathways of β-catenin downregulation might involve caspase-mediated cleavage, or inhibition of guanosine 3′,5′-cyclic monophosphate (cGMP) phosphodiesterase, leading to increased cGMP levels and downregulation of β-catenin, possibly via protein kinase G phosphorylation. Also, NSAIDs may directly inhibit the translocation of β-catenin to the nucleus.

Taken together, our findings demonstrate that NSAIDs suppress TCF-mediated transcription, presumably by preventing nuclear accumulation of active β-catenin. Our observations provide insights in the pathways involved in chemoprevention of NSAIDs and could advance the design of better chemopreventive drugs.

References

NSAIDS target nuclear β-catenin