5-Aminosalicylic Acid Given in the Remission Stage of Colitis Suppresses Colitis-Associated Cancer in a Mouse Colitis Model

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Abstract

Purpose: The risk of colorectal cancer is increased in patients with inflammatory bowel diseases, especially those with ulcerative colitis (UC). Although 5-aminosalicylic acid (5-ASA) is widely used in the treatment of UC to suppress the colitic inflammation, no studies have been conducted to examine the chemopreventive effect of 5-ASA, given in the remission phase of colitis, against colitis-associated cancer using animal models. We therefore investigated the possible inhibition by peroxisome proliferator-activated receptor-γ (PPARγ) ligands and 5-ASA of colitis-associated colon carcinogenesis in a mouse model.

Experimental Design: A dextran sodium sulfate/azoxymethane-induced mouse colon cancer model was used, and the chemopreventive effects of 5-ASA and PPARγ ligands, given in the remission phase of colitis, against colitis-related colon carcinogenesis, were evaluated.

Results: The number of neoplasms in the mice treated with 5-ASA was significantly lower than that in the control mice. In addition, the size of the neoplasms in treated mice was also significantly smaller than that in the control mice. In contrast, no significant suppression in the number or size of the tumors was observed in the mice treated with PPARγ ligands. The proliferating cell nuclear antigen-labeling index in the tumor cells of the 5-ASA-treated mice was significantly smaller than that in the control, indicating that 5-ASA reduced tumor cell proliferation.

Conclusion: Our results revealed that 5-ASA given in the remission phase of colitis significantly suppressed the development of colitis-associated cancer in a mouse model, which indicates the clinical importance of adopting chemopreventive strategies even in UC patients in remission.

In recent decades, the prevalence of inflammatory bowel disease, i.e., ulcerative colitis (UC) and Crohn’s disease has been increasing annually throughout the world. One possible reason is that patients with inflammatory bowel disease survive longer than before because of advances in treatments. On the other hand, the risk of colorectal cancer in these patients, so-called colitis-associated cancer, has also increased, especially in cases of UC. Colitis-associated cancer is believed to be a result of chronic inflammation. A recent meta-analysis has estimated the incidence rate of colitis-associated cancer at 7 per 1,000 person-years and 12 per 1,000 person-years in the second and third decades of UC, respectively (1). Therefore, attempts at prevention of colitis-associated cancer in this high-risk group of patients are considered to be important.

Recent clinical reports have suggested that treatment of UC patients with 5-aminosalicylic acid (5-ASA) might reduce the incidence of colitis-associated cancer (2, 3). Although these reports suggest that treatment of UC patients with 5-ASA may be useful for the prevention of colitis-associated cancer, the precise chemopreventive effects have not been elucidated yet. Therefore, it is considered important that the chemopreventive effect of 5-ASA and the mechanisms underlying the chemoprevention of colitis-associated cancer by 5-ASA have been investigated using animal models. Although 5-ASA is widely used in the treatment of UC to suppress colonic inflammation, no studies have been conducted to examine the chemoprevention by 5-ASA, given in the remission phase of colitis, against colitis-associated cancer using animal models. In addition, the pathogenesis of inflammatory bowel disease–related colitic cancer is still unclear, although various studies using animal...
models have been conducted to investigate the pathogenesis (4–6). Dextran sodium sulfate (DSS) is the most widely used chemical to induce colitis (6), and the DSS-induced colitis model is also expected to be useful for the study of inflammatory bowel disease–related colitis-associated cancer. A relationship between the severity of DSS-induced inflammation and colorectal carcinogenesis, similar to that between human UC-associated dysplasia and the cancer histopathology, has been reported (5). However, the development of these DSS-induced colitis-associated cancer models need long experimental periods or repeated administration of DSS (5). In contrast, several groups have reported that exposure of mice to a single dose of azoxymethane followed by 1 week’s treatment with 2% DSS could induce colonic epithelial malignancy after 6 to 20 weeks. Therefore, the azoxymethane/DSS experimental animal models are useful models for the investigation of carcinogenesis in human UC patients.

Previously, our group reported that peroxisome proliferator–activated receptor γ (PPARγ) ligands reduced colorectal tumor formation in a mouse model of colon carcinogenesis (7). PPARγ, a nuclear hormone receptor, serves as a strong link between lipid metabolism and the regulation of gene transcription (8). PPARγ is known to regulate growth arrest and terminal differentiation of adipocytes (9). In addition, PPARγ is expressed in various organs, including adipose tissue, breast epithelium, small intestine, lungs, and colon (10), and is also up-regulated in various types of cancer cells. Therefore, we conducted this study to investigate whether PPARγ ligands and 5-ASA might inhibit colitis-associated colon carcinogenesis using the DSS/azoxymethane–induced mouse colon cancer model: we evaluated the chemopreventive effects on colitis-related colon carcinogenesis in the remission stage after the induction of colitis, although in many previous studies, the drugs were given before the induction of colitis.

**Materials and Methods**

**Chemicals and animals.** All mice were treated humanely in accordance with the NIH and AERI-BBRI Animal Care and Use Committee guidelines. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yokohama City University School of Medicine. Five-week-old Crl:CD-1 (ICR-1) male mice were purchased from Charles River Japan, Inc. Azoxymethane was purchased from Sigma. DSS with a molecular weight of 40,000 was purchased from MP Biomedicals. The two different PPARγ ligands, pioglitazone and rosiglitazone, were kindly provided by Takeda Chemical Industries, Ltd. (Tokyo, Japan) and GlaxoSmithKline (BN, United Kingdom), respectively. 5-ASA was kindly provided by Nisshin Kyorin Pharmaceutical Co., Ltd. (Tokyo, Japan). The dose levels of the PPARγ ligands were determined on the basis of the results of our previous studies (7).

**Induction of colitis-associated cancer in the mouse model.** Male ICR-1 mice were given a first i.p. injection of azoxymethane (10 mg/kg) on day 0 (see Fig. 1). Seven days after the azoxymethane injection, the mice were given 2% DSS in the drinking water for 7 days. One week after the discontinuation of DSS administration, the mice were given a second i.p. injection of azoxymethane (5 mg/kg). Then, 7 days after the second azoxymethane injection, the mice were again given 2% DSS in the drinking water for 7 days. Two weeks later, the mice were randomly divided into four groups: group 1 was fed a diet without PPARγ ligands or 5-ASA; groups 2 to 4 were fed diets with the PPARγ ligands pioglitazone (25 mg/kg, group 2) or rosiglitazone (25 mg/kg, group 3), or 5-ASA (100 mg/kg, group 4) for 49 days until sacrifice. All mice were sacrificed at the end of the study (day 98; Fig. 1).

![Fig. 1. Experimental protocol for investigating the chemopreventive effects of PPARγ ligands and 5-ASA in a mouse model of colitic cancer. Group 1: Control, 1 wk after the first i.p. azoxymethane (10 mg/kg) injection, the mice were given 2% DSS in drinking water for 7 d. then 1 wk after the second i.p. azoxymethane (5 mg/kg) injection, the mice were again given 2% DSS in the drinking water for 7 d. Group 2: Pio, treated with pioglitazone (25 mg/kg). Group 3: Rosi, treated with rosiglitazone (25 mg/kg). Group 4: 5-ASA, treated with 5-ASA (100 mg/kg).](Cancer Susceptibility and Prevention)
Assessment of colitis. Body weight, the presence of blood in the feces, and stool consistency were recorded daily for each animal. These variables were used to calculate the average daily disease activity index for each animal, as previously described (11). The disease activity index has been shown to be well-correlated with the colon tissue damage score.

Histopathologic analysis. The histopathologic alterations in the colon were examined on H&E-stained sections. A pathologist (Y. Nagashima) diagnosed the colonic neoplasms according to a previously described method (12).

Immunohistochemistry. Immunohistochemistry for proliferating cell nuclear antigen (PCNA) and β-catenin was done. For PCNA immunohistochemistry, we used a PCNA staining kit (ZYMED Laboratories) in accordance with the manufacturer’s instructions. For β-catenin immunohistochemical analysis, we used a monoclonal antibody directed against β-catenin (1:1,200 dilution; BD Transduction Laboratories) and a Vectastain ABC kit (Vector Laboratories).

The PCNA labeling index was expressed as the percentage of cells showing positive staining for PCNA relative to the total number of cells. At least five representative areas in a section were selected by light microscope examination at 400-fold magnification and a minimum of 3,000 tumor cells were counted (13).

Statistical analysis. Statistical analysis of the changes in the body weights of the mice, number of neoplasms, and size of neoplasms were done using a $\chi^2$ test. Differences were considered significant when at $P < 0.05$.

### Table 1. Number and size of tumors

<table>
<thead>
<tr>
<th>Group</th>
<th>$n$</th>
<th>Body weight (g)</th>
<th>No. of tumors</th>
<th>No. of tumors/mouse</th>
<th>Size of tumors (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) Control</td>
<td>8</td>
<td>39.0 ± 1.3*</td>
<td>70</td>
<td>8.5 ± 4.0*</td>
<td>3.3 ± 1.5*</td>
</tr>
<tr>
<td>(2) Pioglitazone</td>
<td>6</td>
<td>40.8 ± 1.0*</td>
<td>57</td>
<td>9.5 ± 4.0*</td>
<td>3.6 ± 2.1*</td>
</tr>
<tr>
<td>(3) Rosiglitazone</td>
<td>6</td>
<td>39.5 ± 2.4*</td>
<td>58</td>
<td>8.8 ± 4.9*</td>
<td>3.8 ± 1.8*</td>
</tr>
<tr>
<td>(4) 5-ASA</td>
<td>8</td>
<td>40.0 ± 4.7*</td>
<td>38</td>
<td>4.0 ± 2.8*</td>
<td>2.4 ± 1.1*</td>
</tr>
</tbody>
</table>

NOTE: A total of 33 male ICR-1 mice were divided into control and experimental groups. The body weights of the mice did not change significantly. As clearly shown, 5-ASA suppressed the formation of neoplasms ($P < 0.05$). The size of the neoplasms in the colon of mice fed the diet containing 5-ASA was smaller than that in the colon specimens from other groups ($P < 0.01$).

*Mean ± SD.

Fig. 3. Microscopic examination of H&E-stained colon sections and β-catenin staining of the tumors. Left, H&E staining (original magnification, ×40). Middle, β-catenin staining (original magnification, ×40). Right, β-catenin staining (original magnification, ×400).
Results

Disease activity index and body weight changes. The disease activity index was estimated according to the method described previously (11). The disease activity increased dramatically immediately after DSS treatment, to decrease again by 1 week after the second treatment with DSS (Fig. 2). The body weights of the mice measured at the end of the study period are shown in Table 1. No significant changes in the mean body weights of the mice was observed in any of the groups.

5-ASA, but not the PPARγ ligands, suppressed tumor formation in the azoxymethane/DSS mice. The number and size of the tumors in the colon specimens obtained from the mice of each group at the end of the study period are shown in Table 1. The number of neoplasms in the colon specimens from the mice treated with 5-ASA (group 4) was significantly lower than that in the colon of the control animals (group 1, \(P < 0.05\)). In addition, the size of the neoplasms in the colon specimens of the mice treated with 5-ASA were significantly smaller than that in the colon of the control mice. In contrast, no significant decrease of the tumor number and size was observed in the colon specimens from the mice treated with the PPARγ ligands.

Pathologic findings. H&E-stained sections of the tumors from all the groups are shown in Fig. 3. Macroscopically, nodular, polypoid tumors were observed in the middle and distal colon in all the groups. No differences in the morphologic characteristics of the tumors were observed among the groups.

Immunochemistry for PCNA and β-catenin. The PCNA labeling index (normal epithelial cells and tumor cells) values are shown in Table 2. The PCNA labeling index in the epithelial cells did not differ significantly among the groups (\(P > 0.05\); Supplementary Figure). However, the PCNA labeling index in

<table>
<thead>
<tr>
<th>Group no., treatment</th>
<th>PCNA-labeling index</th>
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<tr>
<td></td>
<td>Non–tumor cells (%)</td>
</tr>
<tr>
<td>(1) Control</td>
<td>26.10 ± 5.31*</td>
</tr>
<tr>
<td>(2) Pioglitazone</td>
<td>20.94 ± 8.08*</td>
</tr>
<tr>
<td>(3) Rosiglitazone</td>
<td>18.25 ± 2.40*</td>
</tr>
<tr>
<td>(4) 5-ASA</td>
<td>23.04 ± 6.34*</td>
</tr>
</tbody>
</table>

NOTE: The PCNA labeling index is expressed as the percentage of cells showing positive staining for PCNA relative to the total number of cells examined. At least five representative areas in a section were selected by light-microscopic examination at 400-fold magnification and a minimum of 3,000 tumor cells were counted. No significant differences in the PCNA labeling index of non–tumor cells was observed among the groups (\(P > 0.05\)). 5-ASA significantly decreased the PCNA labeling index in the tumor cells (\(P < 0.05\)).

*A mean ± SD.
the tumor cells in group 4 (5-ASA treatment), but not groups 2 or 3 (treated with the PPARγ ligands), was significantly smaller than that in group 1 (control, P < 0.05). Typical microscopic photographs are shown in Fig. 4. Strong β-catenin expression was seen in the nucleus and cytoplasm of the tumor cells, but there were no significant differences among the tumors in the four groups (Fig. 3, middle and right).

**Discussion**

In the present study, we clearly showed the chemopreventive effect of 5-ASA against colitis-associated cancer in the remission stage. In contrast, the PPARγ ligands showed no suppressive effect against neoplasm formation. Previously, our group and others have reported that the PPARγ ligands effectively inhibited the colonic inflammation associated with DSS administration, trinitrobenzene sulfonic acid–induced colitis, as well as aberrant crypt foci formation in many animal models (5, 14, 15). However, most of these reports examined the preventive effect of PPARγ ligand therapy, i.e., the PPARγ ligands were given before the induction of inflammation (pretreatment). In the present study, we gave the PPARγ ligands and 5-ASA 2 weeks after the end of DSS treatment. Therefore, all mice were in the remission stage after the induction of colitis. The remission stage was also confirmed by the disease activity index, as shown in Fig. 2 and in the Supplementary Table. As PPARγ ligands have anti-inflammatory actions, their antitumor effects against colitis-associated cancer shown in previous studies might be the result of their anti-inflammatory effects in colitis-associated cancer (16). This might also be the reason why the PPARγ ligands showed no suppressive effect against tumor development in our present study.

In the present study, 5-ASA markedly suppressed the development of colitic cancer. Although the PCNA labeling index in the non–tumor colonic epithelium did not differ significantly among the groups, only 5-ASA alone significantly suppressed the PCNA labeling index in the tumor cells. These results suggest that 5-ASA may reduce tumor cell but not normal epithelial cell proliferation. Further investigations will be required to clarify the detailed mechanisms.

In conclusion, we found that only 5-ASA given in the remission stage of colitis significantly suppressed the development of colitis-associated cancer in a mouse model. Our results showed the clinical importance of adopting a chemopreventive strategy in UC patients in remission.

**References**

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