

Induction of Apoptosis by Sulindac in Azoxymethane-induced Possible Colonic Premalignant Lesions in Rats

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We have reported that β -catenin-accumulated crypts (BCAC), which do not have the appearance of aberrant crypt foci (ACF) are possible colonic premalignant lesions in rats. Suppression of the occurrence and advancement of such lesions should have critical relevance to cancer prevention. This study examined whether sulindac, a chemopreventive nonsteroidal anti-inflammatory drug is able to induce apoptosis in such premalignant lesions. At 6 weeks of age, rats groups 1–3 were given azoxymethane (AOM) (15 mg/kg-body weight) once weekly for 3 weeks. Two groups were given sulindac in the diet (200 and 400 ppm), starting at 9 weeks of age. The rats were sacrificed at the termination, and the colons were carefully examined. The incidence and crypt multiplicity of BCAC and ACF were significantly less than those of the control group. The effect of sulindac on the expression of BCAC was greater than that on ACF. Exposure to sulindac significantly increased the apoptotic index (terminal deoxynucleotide transferase dUTP nick-end labeling (TUNEL)-positive cells) in BCAC. However, no significant increase of the index was found in the case of ACF. These results suggest that the chemopreventive effect of sulindac in rats is related to the induction of apoptosis in premalignant lesions. Our results also provide additional evidence that BCAC are premalignant lesions in colon carcinogenesis in rodents.

Key words: Apoptosis — β -Catenin-accumulated crypts — Aberrant crypt foci — Sulindac — Chemoprevention

Colorectal cancer is a malignancy with one of the highest incidences and mortality rates of human neoplasms.¹⁾ Chemoprevention embraces the concept that non-carcinogenic synthetic chemicals or naturally occurring products can inhibit the process of carcinogenesis. Epidemiological studies have revealed that chronic ingestion of nonsteroidal anti-inflammatory drugs (NSAIDs), such as aspirin, reduces the risk of colorectal cancer and decreases the incidence of colon adenomas.²⁾ Sulindac, one of the NSAIDs, has been shown to reduce the number and size of colonic polyps in patients with familial adenomatous polyposis.³⁾ Animal studies also proved that NSAIDs protect against chemically induced colon carcinogenesis.^{4,5)} Since large numbers of chemopreventive agents, including NSAIDs, may exist, they may act through a range of mechanisms that may interact synergistically or otherwise. Although the precise mechanism by which NSAIDs inhibit colon tumorigenesis is not fully known, available data support the hypothesis that they are involved in the inhibition of prostaglandin (PG) synthesis through the modulation of cyclooxygenase (COX) activity.^{6–8)}

Sulindac is a potent inhibitor of both COX-1 and COX-2 enzymes.⁹⁾ The inhibitory effect of sulindac on the spontaneous occurrence of intestinal tumors in rodents is sug-

gested to be associated with a decrease in mucosal PGE2 levels.¹⁰⁾ It is reported that sulindac inhibits prostanoid synthesis by acting on the COX enzyme activity and modulates cell proliferation through other pathways.^{8,11)}

Studies on prevention of colon cancer require the development of biomarkers suitable for the rapid evaluation of potential chemopreventive agents and nutritional agents. Aberrant crypt foci (ACF) are regarded as putative pre-neoplastic lesions in rodents and are used as an intermediate biomarker to evaluate chemopreventive efficacy.¹²⁾ Recently, we have reported the presence of β -catenin accumulated crypts (BCAC), which appear soon after carcinogen exposure, like ACF, and which are morphologically distinguishable from ACF.¹³⁾ The properties of the early-appearing crypts at the molecular and cellular levels suggest that they are more likely to be direct precursors of colon tumors than are classical ACF in rats,¹⁴⁾ and such lesions could be a novel biomarker to evaluate chemopreventive efficacy.¹⁵⁾ It is important to note that an intermediate biomarker for studies on cancer chemoprevention should be closely associated with the pathway of carcinogenesis.¹⁶⁾ Results of recent studies support the idea that tumor growth *in vivo* depends on evasion of homeostatic control that leads to induction of cell death by apoptosis. NSAIDs, which are regarded as promising chemopreventive agents, are suggested to generate cellular apopto-

sis.^{6,17} However, the findings on NSAIDs, including sulindac, have been obtained mostly in *in vitro* studies. Meanwhile, it has been reported that the efficacy of various antitumor agents is related to the intrinsic ability of the target tumor cells to respond to these agents with induction of apoptosis.¹⁸ Conversely, transformation of colorectal epithelia to adenomas and adenocarcinomas is suggested to be associated with a progressive inhibition of apoptosis.¹⁹

It is important to know whether chemopreventive agents induce apoptosis in premalignant lesions or not. This study was designed to examine the potential of sulindac, a NSAID, for the induction of apoptosis and for the control of proliferative kinetics in the premalignant lesions of colon cancers in rats.

MATERIALS AND METHODS

Animals and diet Male F344 rats (Shizuoka SLC Co., Shizuoka), 4 weeks old, were used. All animals were housed wire cages (3 or 4 rats/cage) with free access to drinking water and CE-2 basal diet (CLEA Japan Inc., Tokyo) under controlled conditions of humidity (50±10%), lighting (12 h light/dark cycle) and temperature (23±2°C). They were quarantined for 2 weeks and randomized into experimental and control groups for this study. Powdered CE-2 diet (345.2 cal) was used as the basal diet throughout the study.

Chemicals and experimental procedure Thirty-two rats were divided into four groups. Sulindac and azoxymethane (AOM) were purchased from Sigma Chemical Co. (St. Louis, MO). Animals were given subcutaneous injections of AOM (15 mg/kg-body weight) once a week for 3 weeks and sacrificed at 10 weeks after the first injection of the carcinogen. At sacrifice, the colons were removed, cut open along the longitudinal axis and fixed in 10% buffered formalin for 24 h. Afterward, the colonic mucosa in 3 cm of segments from distal and middle portion were used for analysis of colonic lesions.

Determination of ACF and β -catenin accumulated crypts For the ACF analysis, colon sections were dripped into a 0.2% methylene blue solution for 30 s, and washed in saline. Using a light microscope at a magnification of $\times 40$, ACF were distinguished from the surrounding normal crypts by their increased size, increased distance from lamina to basal surface of cells and discernible pericryptal zone. The parameters used to assess the aberrant crypts were their occurrence and crypt multiplicity. Crypt multiplicity was determined as the number of crypts in each focus. BCAC were detected as described previously.^{13,14} Colonic mucosal sections were examined using an *en face* preparation and 3- to 5- μ m thick serial sections. For immunohistochemical analysis, the labeled streptavidin biotin method was performed using primary antibodies

against β -catenin (diluted 1:1000; Transduction Laboratories, Lexington, KY). Two pathologists evaluated these immunoreactivities independently.

AgNORs (silver-stained nucleolar organizer regions) AgNORs staining, a marker of cell proliferation, was performed according to the method described previously,²⁰ with minor modifications. AgNOR counts/nucleus were scored on AgNOR-stained sections by microscopy at a magnification of $\times 400$.

TUNEL staining (terminal deoxynucleotide transferase dUTP nick-end labeling) TUNEL staining was performed as described previously with some modifications.²¹ After incubation with 20- μ g/ml proteinase K (Sigma), the serial sections used for hematoxylin and eosin staining was immersed in TDT buffer (30 mmol/liter Trizma base, pH 7.2, 140 mmol/liter sodium cacodylate, 1 mmol/liter cobalt chloride). Biotinylated dUTP (Boehringer Mannheim GmbH, Mannheim, Germany) and TDT (Boehringer Mannheim GmbH) were diluted in TDT buffer at a concentration of 0.8 nmol and 0.15 EU/ μ l, respectively. The solution was placed on the sections, and then incubated at 37°C for 60 min. The sections were covered with streptavidin peroxidase. Finally, counterstaining was done using Mayer's hematoxylin. The percentage of TUNEL-positive cells was determined by measuring the number of epithelial cells possessing dark-brown-staining nuclei, as a proportion of the entire epithelial cell number of each lesion on the section. Normal crypts, adjacent to ACF and β -catenin accumulated crypts, were chosen randomly, and about 14 000 cells were counted by an observer blinded to the animal treatment group.

RESULTS

Incidences and multiplicity The incidences of ACF and BCAC are shown in Table I. In group 1, the frequencies of ACF and BCAC were respectively 10.42±2.27 and 4.20±1.83/cm² colonic mucosa. The dietary exposure to sulindac caused a dose-dependent reduction in the incidence of BCAC: 3.23±1.61 in group 2 (23.1% inhibition) and 2.39±0.97 in group 3 (43.1% inhibition, $P < 0.002$; Welch's *t* test). Sulindac was also found to reduce the incidence of ACF: 8.78±3.61 in group 2 (15.7% inhibition) and 6.45±1.67 in group 3 (38.1% inhibition).

As to crypt multiplicity, a significant decrease in the number of crypts/lesion in group 3 was found in both BCAC and ACF when compared with that in group 1 ($P < 0.01$; Welch's *t* test and $P < 0.01$; Student's *t* test, respectively) (Table II). The inhibitory effect on BCAC exceeded that on ACF.

AgNOR count/nucleus The data for numbers of AgNORs/nucleus of the epithelium in ACF, BCAC and adjacent normal crypts are shown in Table III. The mean AgNOR count/nucleus was greatest in BCAC in each

Table I. Incidence of Aberrant Crypt Foci and β -Catenin-accumulated Crypts

Group no.	Treatment	No. of segments examined	No. of ACF/cm ² colonic mucosa	No. of BCAC/cm ² colonic mucosa
1	AOM+basal diet	16	10.42±2.27 ^{a)}	4.20±1.83
2	AOM→200 ppm sulindac	16	8.78±3.61 (15.7)	3.23±1.61 (23.1)
3	AOM→400 ppm sulindac	16	6.45±1.67 ^{b)} (38.1)	2.39±0.97 ^{c)} (43.1)
4	No treatment	16	0	0

a) Values are mean±SD.

b) Significantly different from group 1 by Student's *t* test ($P<0.001$).

c) Significantly different from group 1 by Welch's *t* test ($P<0.002$).

Number in parentheses, percentage of inhibition compared to the respective control groups.

Table II. Crypt Multiplicity of Aberrant Crypt Foci and β -Catenin-accumulated Crypts

Group no.	Treatment	ACF		BCAC	
		No. of ACF examined	No. of crypts/ACF	No. of BCAC examined	No. of crypts/BCAC
1	AOM+basal diet	223	4.43±2.06 ^{a)}	88	3.60±3.23
2	AOM→200 ppm sulindac	254	4.35±2.07 (1.8)	91	3.08±2.23 (14.4)
3	AOM→400 ppm sulindac	201	3.81±1.84 ^{b)} (14.0)	76	2.54±1.77 ^{c)} (29.4)

a) Values are mean±SD.

b) Significantly different from group 1 by Student's *t* test ($P<0.01$).

c) Significantly different from group 1 by Welch's *t* test ($P<0.01$).

Number in parentheses, percentage of inhibition compared to the respective control groups.

Table III. Mean Number of AgNOR/nucleus

Group no.	Treatment	No. of normal crypts examined	Mean no. of counts in normal crypts	No. of ACF examined	Mean no. of counts in ACF	No. of BCAC examined	Mean no. of counts in BCAC
1	AOM+basal diet	15	1.68±0.35 ^{a)}	15	2.27±0.30	15	3.41±0.40
2	AOM→200 ppm sulindac	15	1.63±0.27	15	1.87±0.30 ^{b)} (17.6)	15	2.19±0.47 ^{c)} (35.7)
3	AOM→400 ppm sulindac	15	1.57±0.37	15	1.74±0.37 ^{c)} (23.3)	15	1.80±0.44 ^{c)} (47.2)

a) Values are mean±SD.

b) Significantly different from group 1 by Student's *t* test ($P<0.002$).

c) Significantly different from group 1 by Student's *t* test ($P<0.001$).

Number in parentheses, percentage decrease compared to the respective control groups.

group. The numbers in ACF and BCAC of groups 2 and 3 were significantly smaller than those in group 1 ($P<0.01$; Student's *t* test). The degree of reduction of AgNORs count/nucleus in BCAC exceeded that seen in ACF.

Apoptotic index The results on apoptotic index in each lesion are shown in Fig. 1. Apoptotic index values in BCAC in rats of groups given 200 and 400 ppm sulindac together with AOM were significantly larger than that of rats with AOM alone ($P<0.0003$ and $P<0.0003$, respectively; Welch's *t* test). The values of ACF and adjacent

normal crypts in the experimental groups were not significantly different from those of the control group.

DISCUSSION

Although chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, their ability to induce apoptosis and to suppress proliferative activity may represent a unifying concept for the mechanism of chemoprevention. Understanding the modes

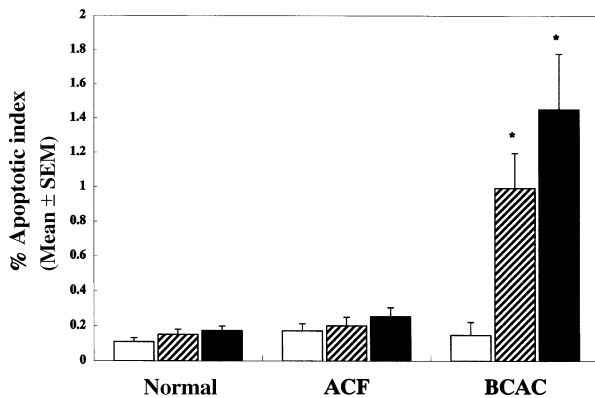


Fig. 1. Apoptotic index values of normal colonic crypts, ACF and BCAC on AOM-induced colonic mucosa in male F344 rats. In BCAC, mean apoptotic index values in groups 2 and 3 were significantly larger than that in group 1 (*, $P < 0.0003$; Welch's *t* test). □, group 1 AOM alone; ▨, group 2 sulindac (200 ppm); ■, group 3 sulindac (400 ppm).

of action of these compounds should provide useful information for their possible application in cancer prevention. This study *in vivo* demonstrated that dietary administration of sulindac, which was proved to inhibit colon carcinogenesis, reduced the incidence and crypt multiplicity of both types of premalignant lesions.⁴⁾ Our results suggest that the inhibitory effects of sulindac may be attributable to induction of apoptosis as well as suppression of cell proliferation. It has been reported that sulindac sulfide, in addition to its antiproliferative effects, induces apoptosis in HT-29 colon adenocarcinoma cells.²²⁾ There are also several studies showing that sulindac causes increased apoptosis in colonic tumors.^{6,7)} Additionally, induction of apoptosis by sulindac was reported in cases of familial adenomatous polyposis.²³⁾ Such data suggest that sulindac-induced apoptosis in the late promotion or progression stage is clinically relevant in the treatment of colon neoplasms. In the present study, sulindac induced cellular apoptosis in the early appearing premalignant lesions. It is important to note that sulindac is not an antitumor agent, but a chemopreventive agent. Thus, the data represent important evidence concerning the mode of action of NSAIDs and the role of apoptosis in the early phase of carcinogenesis from the viewpoint of cancer prevention.

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Our previous studies characterizing BCAC at the molecular and cellular levels have implicated the early appearing crypts as intermediate lesions leading to colon cancer.¹⁴⁾ BCAC possesses a characteristic morphology with increased proliferation kinetics and frequent mutations of β -catenin gene, which is regarded as a key gene in the process of colon cancer development. Interestingly, BCAC are basically independent of classical ACF, which have also been described as early appearing preneoplastic lesions for colon cancers. Proliferative activity of crypts with accumulation of β -catenin is stronger than in ACF, suggesting that BCAC are more likely to progress to malignant transformation. Intriguingly, administration of sulindac caused an increased apoptotic index in the BCAC, whereas there was no clear increase in the case of ACF. The levels of the apoptotic indices recognized in BCAC in rats of group 3 were 10-fold higher than those in group 1. Sulindac is reported to induce apoptosis in colonic cancers induced by AOM in rats.⁷⁾ Accordingly, our results may represent additional evidence that the biological characteristics of BCAC are similar to those of colon cancer and that BCAC are a possible direct precursor of the malignancy. It is necessary to explore BCAC in human colon to clarify the mechanism of human colon carcinogenesis, and a study is in progress.

In conclusion, we have shown that dietary exposure to sulindac at the postinitiation phase suppresses the incidence and advancement of premalignant lesions for colon cancer. Sulindac caused an increased apoptotic index in BCAC, whereas no significant increase of the index occurred in ACF. To our knowledge, this is the first study to demonstrate induction of apoptosis in premalignant lesions for large bowel malignancy in an animal model. Such findings suggest that the induction of apoptosis in these early appearing lesions is one of the key actions of chemopreventive agents, particularly those like NSAIDs.

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