Suppression of Occurrence and Advancement of β-Catenin-accumulated Crypts, Possible Premalignant Lesions of Colon Cancer, by Selective Cyclooxygenase-2 Inhibitor, Celecoxib

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Suppression of occurrence and advancement of premalignant lesions is important for cancer prevention. Our previous studies clarified that β-catenin-accumulated crypts, independent of aberrant crypt foci (ACF), are probably direct precursors of colon cancers in rats. Here we investigated the effects of a selective cyclooxygenase-2 inhibitor, celecoxib, on the development of β-catenin-accumulated crypts in comparison with those on ACF. Male F344 rats were divided into 4 groups. Groups 1–3 were administered azoxymethane (AOM) s.c. at a dose of 15 mg/kg body weight, once weekly for 3 weeks to induce β-catenin-accumulated crypts. Groups 2 and 3 also received experimental diet containing celecoxib (500 and 1500 ppm, respectively) for 8 weeks, starting a week before the first dosing of AOM. At termination, the frequency and crypt multiplicity (number of crypts/lesion) of β-catenin-accumulated crypts of groups 2 and 3 were significantly less than that of group 1. Furthermore, numbers of silver-stained nucleolar organizer regions (AgNORs)/nucleus in β-catenin-accumulated crypts were also decreased by exposure to celecoxib. In this study, celecoxib had greater effects on the frequency and growth of β-catenin-accumulated crypts than on those of ACF. These findings represent additional evidence that β-catenin-accumulated crypts are premalignant lesions of colon cancer. The results also suggest that β-catenin-accumulated crypts could be a novel target for evaluation of possible chemopreventive agents against colon carcinogenesis, and indicate that possible chemopreventive effects of celecoxib on the initial stage of colon carcinogenesis may be related to modulation of cell proliferation activity in such early lesions.

Key words: Chemoprevention — Celecoxib — Colon carcinogenesis — Premalignant lesion — Rat

Colorectal cancer is one of the leading causes of cancer deaths all over the world and is therefore a major public health problem. During the last decade, numerous studies including molecular analysis have focused on discovery of possible chemopreventive agents against colon carcinogenesis. Several epidemiological and clinical studies have suggested an inverse association between risk of colon cancer and intake of nonsteroidal anti-inflammatory drugs (NSAIDs).[1, 2] Studies in laboratory animals have also provided convincing evidence that exposure to various NSAIDs inhibits chemically induced colon carcinogenesis.[3, 4] Although the precise mechanism by which NSAIDs prevent colon carcinogenesis is not clear, it may involve the inhibition of arachidonic acid metabolism by two cyclooxygenase (COX) isozymes, COX-1 and COX-2. It is known that commonly used NSAIDs inhibit the activity of both isozymes of COX. However, recent studies have focused on specific COX-2 inhibitors as potent chemopreventive agents for colorectal cancers.[5, 6] Among COX-2 inhibitors, celecoxib is now regarded as the most

prominent agent for the suppression of the development of colon tumors.[5, 7]

Colon carcinogenesis is a representative multistep tumorigenesis with accumulating genetic alterations.[8] Alterations in the *APC* or *β-catenin* gene are regarded as early and critical events during colon carcinogenesis and are therefore considered to play a gatekeeper role in development of colon cancers in both humans and rodents.[9–11] Such mutations are suggested to result in accumulation of β-catenin protein.[12, 13] Recently, β-catenin has been proved to function as a transcriptional activator when complexed with members of the T cell factor (Tcf) family of DNA binding proteins.[14, 15] Furthermore, target genes of the β-catenin signaling pathway were identified as growth-promoting genes, such as c-*myc* and *cyclin D1*,[16, 17] suggesting that the pathway is oncogenic.

Previously, we found the presence of altered crypts with excessive β-catenin in rat colonic mucosa that was predisposed to colon cancer.[18, 19] In addition to the accumulation of the oncogenic β-catenin protein, such crypts harbored frequent mutations in the *β-catenin* gene.[18] Histological observations also showed that β-catenin-accumulated crypts exhibit dysplasia, a hallmark of malignant potential,

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and their size increased with time after carcinogen exposure.[19] These findings clearly indicate that such early appearing lesions are premalignant lesions of colon cancer in rats. Interestingly, the β-catenin-accumulated crypts are generally independent of aberrant crypt foci (ACF) that were described as early appearing lesions and putative preneoplastic lesions of colon cancer.[20] Importantly, the proliferative activity of crypts with accumulation of β-catenin is significantly higher than that of ACF.[19] Therefore, it is reasonable to conclude that β-catenin-accumulated crypts are more likely to progress to malignant transformation than ACF.

The major aim of this study is to investigate the modifying effects of a specific COX-2 inhibitor, celecoxib, on β-catenin-accumulated crypts. Although, there are a number of reports demonstrating that chemopreventive agents, including celecoxib,[21] inhibit the formation of ACF, this study seems to be the first report to investigate the effect of chemopreventive agent on the possible direct precursor of colon cancer.

MATERIALS AND METHODS

Animals, carcinogen, test chemicals and diets Male F344 rats (Shizuoka Laboratory Animal Center, Shizuoka) aged 4 weeks were used. All animals were housed in wire cages (3 or 4 rats/cage) with free access to drinking water and basal diet, CE-2 (CLEA Japan Inc., Tokyo), under controlled conditions of humidity (50±10%), lighting (12-h light/dark cycle) and temperature (23±2°C). A total of 28 rats were quarantined for 7 days and randomized into experimental and control groups (Fig. 1). Azoxymethane (AOM) was purchased from Sigma Chemical Co., St. Louis, MO. Celecoxib (SC-58635; 4-[5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene-sulfonamide) was

kindly supplied by Searle Research and Development (St. Louis, MO). Experimental diets were made by mixing test chemicals in powdered basal diet CE-2 at a concentration of 500 or 1500 ppm. The dose levels of test compounds were determined on the basis of previous reports.[5, 7]

Experimental procedure A total of 28 male F344 rats were divided into 4 groups as shown in Fig. 1. Groups 1 through 3 were initiated with AOM by 3 weekly subcutaneous injections (15 mg/kg body weight). Rats in groups 2 and 3 were respectively fed the diets containing 500 and 1500 ppm celecoxib, starting one week before the first dose of AOM. Group 4 served as a control. The experiment was terminated 8 weeks after the start and all animals were sacrificed to assess the occurrence and growth of β-catenin-accumulated crypts. Immediately after the sacrifice, colons were removed, cut open along the longitudinal axis, fixed flat in 2% paraformaldehyde in 0.1 M/liter phosphate-buffered saline (pH 7.4) for 24 h at 4°C. Colonic mucosal sections were examined by utilizing an *en face* preparation and 3- to 5-μm thick serial sections. We examined colonic mucosa in three different segments, distal, medium-distal, and proximal-medium, and a total of 112 segments were used for the analysis. The segments were stained with methylene blue and the frequency of ACF was determined. ACFs were distinguished from the surrounding 'normal-appearing' crypts by their increased size. For each case, 20–40 serial sections of crypts were prepared to investigate whole crypts from the mucosal surface to the crypt bottom.[18, 19]

COX-2 immunohistochemistry To determine the localization of COX-2 protein, we performed immunohistochemical analysis. For immunohistochemical analysis, the labeled streptavidin biotin method was performed using an LSAB KIT (DAKO, Glostrup, Denmark) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene, and rehydrated through graded alcohols at room temperature. A 0.05 M Tris-HCl buffer (pH 7.6) was used to prepare solutions and for washes between various steps. Incubations were performed in a humidified chamber. Sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4°C with primary antibodies against rat COX-2 protein (diluted 1:100, Transduction Laboratories, Lexington, KY). For each case, negative controls were performed on serial sections. For the control section, incubation with the primary antibody was omitted. Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with hematoxylin. Immunoreactivities were regarded as positive if apparent stainings were detected in cytoplasm of early appearing lesions.

Determination of β-catenin-accumulated crypts Using specific antibody against β-catenin protein (diluted
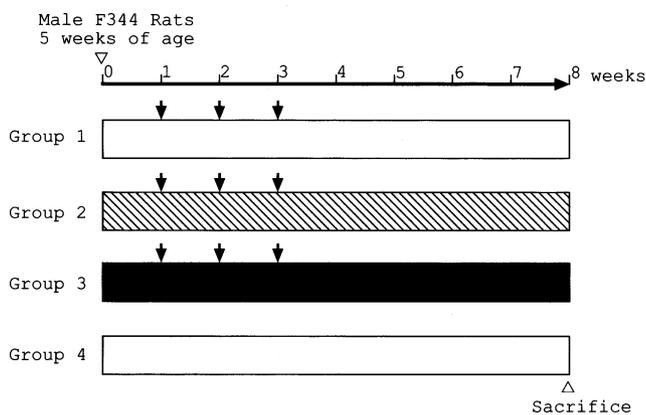

Fig. 1. Experimental protocol. ↓, AOM (15 mg/kg body weight), s.c.; □, basal diet, CE-2; ▨, celecoxib, 500 ppm in diet; ■, celecoxib, 1500 ppm in diet.

1:1000, Transduction Laboratories), immunohistochemistry was performed according to the same procedure as COX-2 immunohistochemistry. Two pathologists evaluated these immunoreactivities independently. At least two sections per preparation were examined for β-catenin immunohistochemistry, and samples were regarded as positive when apparent immunoreactivities in cytoplasm or nucleus were recognized in more than one section. β-Catenin-accumulated crypts were examined for their frequency and crypt multiplicity (number of crypts/lesion) of the lesion.

Silver-stained nucleolar organizer regions (AgNORs) Analysis of AgNORs staining, which is thought to be a biomarker of cell proliferation, was carried out according to the method described previously,[22] with minor modifications. β-Catenin-accumulated crypts in each group were used and two sections from the upper and lower portion of the mucosa were evaluated. AgNORs counts/nucleus were scored on AgNORs stained sections by microscopy at a magnification of ×400.

RESULTS

COX-2 overexpression Six of 10 β-catenin-accumulated crypts and 3 of 10 ACF, as well as stromal cells, showed mild-to-moderate immunopositivity of COX-2 protein. In positive cases, the expression was detected in approximately 50–80% of the total premalignant cells in such early lesions. Adjacent normal crypts revealed no immunostaining of COX-2 except for slight expression in cytoplasm of epithelium located at the crypt base or adjacent to lymph follicles. Immunostaining in β-catenin-accu-

mulated crypts tended to be more evident than in ACF (Fig. 2).

Frequency and crypt multiplicity of β-catenin-accumulated crypts and ACF The incidence of β-catenin-accumulated crypts and ACF is shown in Table I. In group 1, 4.77 ± 1.55 β-catenin-accumulated crypts/cm^2 colonic mucosa and 9.33 ± 4.82 ACF/cm^2 colonic mucosa were present. The dietary administration of celecoxib caused a significant reduction in the incidence of β-catenin-accumulated crypts: 2.51 ± 1.88 in group 2 (47.4% inhibition), 1.45 ± 1.25 in group 3 (69.6% inhibition) (Table I). Celecoxib was also found to reduce the incidence of ACF: 7.37 ± 3.36 in group 2 (21.0% inhibition), 6.20 ± 3.98 in group 3 (33.5% inhibition) (Table I). A significant decrease in the number of crypts/lesion in group 3 was observed in both β-catenin-accumulated crypts and ACF ($P < 0.001$ and $P < 0.004$ by Welch's t test) when compared with those in group 1. The degree of decrease in crypt multiplicity in β-catenin-accumulated crypts (36.1%) was larger than that in ACF (12.5%) (Table II). Diameters of β-catenin-accumulated crypts in group 3 were also smaller than those in group 1 (data not shown). We did not observe β-catenin-accumulated crypts or ACF in group 4.

Regional incidence of β-catenin-accumulated crypts In the present study, to determine regional effects of celecoxib, we examined three different segments of the colon; distal, medium-distal, and proximal-medium. In all segments, celecoxib significantly suppressed the occurrence of β-catenin-accumulated crypts ($P < 0.02$ by Student's t test). However, the decrease in the frequency of β-catenin-accumulated crypts in the proximal-medium segment (75.4% inhibition by 500 ppm celecoxib, and 80.2% inhi-
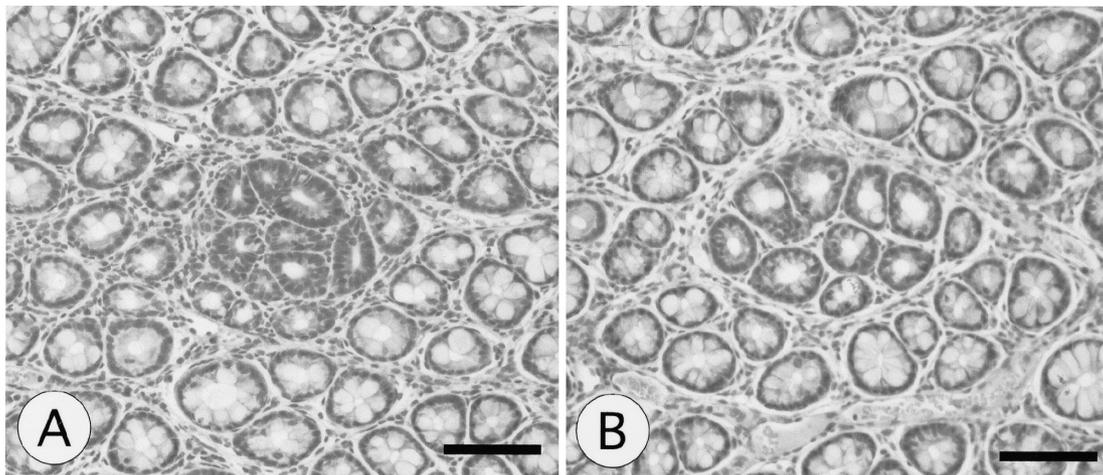

Fig. 2.   Immunohistochemical staining of COX-2 in cancer-predisposed colonic mucosa. Note that immunopositivity for COX-2 protein was detected in cytoplasms of β-catenin-accumulated crypts (A) and aberrant crypt foci (B) as well as stromal cells. Adjacent normal crypts showed no immunostaining of COX-2. Bars, 100 μm.

bition by 1500 ppm celecoxib) exceeded that seen in the medium-distal (40.6% and 62.9%, respectively) or the distal segment (27.0% and 66.6%, respectively) (Table III).

AgNORs count/nucleus in β-catenin-accumulated crypts Previously, we reported an increase of AgNORs count/nucleus of the epithelium in β-catenin-accumulated

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

Group no.	Treatment	No. of segments examined	No. of CAC (lesion) /cm^2 colonic mucosa	No. of ACF (lesion) /cm^2 colonic mucosa
1	AOM alone	21	4.77±1.55[a]	9.33±4.82
2	AOM+500 ppm celecoxib	21	2.51±1.88[b] (47.4)	7.37±3.36 (21.0)
3	AOM+1500 ppm celecoxib	21	1.45±1.25[b] (69.6)	6.20±3.98[c] (33.5)
4	No treatment	21	0	0

CAC, β-catenin-accumulated crypts; ACF, aberrant crypt foci.
a) Mean±SD.
b, *c*) Significantly different from group 1 by Student's *t* test (*b*) $P<0.001$, *c*) $P<0.03$).
Values in parentheses represent percentage inhibition compared to group 1.

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

Group no.	Treatment	No. of CAC (lesion) examined	No. of crypts/CAC	No. of ACF (lesion) examined	No. of crypts/ACF
1	AOM alone	66	4.57±3.00[a]	98	2.96±0.77
2	AOM+500 ppm celecoxib	31	3.87±2.20 (15.3)	86	3.08±0.92
3	AOM+1500 ppm celecoxib	26	2.92±1.49[b] (36.1)	78	2.59±0.87[c] (12.5)

CAC, β-catenin-accumulated crypts; ACF, aberrant crypt foci.
a) Mean±SD.
b) Significantly different from group 1 by Welch's *t* test ($P<0.001$).
c) Significantly different from groups 1 and 2 by Student's *t* test ($P<0.004$).
Values in parentheses represent percentage decrease compared to group 1.

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

A. β-Catenin-accumulated crypts

Group no.	Treatment	No. of segments examined	No. of CAC (lesion)/cm^2 in each segment		
			Distal	Medium-distal	Proximal-medium
1	AOM alone	7	4.67±1.80[a]	5.07±1.32	4.58±1.75
2	AOM+500 ppm celecoxib	7	3.41±2.10 (27.0)	3.01±1.44[c] (40.6)	1.13±1.37[d] (75.4)
3	AOM+1500 ppm celecoxib	7	1.56±1.30[b] (66.6)	1.88±1.46[d] (62.9)	0.90±1.11[e] (80.2)
4	No treatment	7	0	0	0

B. Aberrant crypt foci

Group no.	Treatment	No. of segments examined	No. of ACF (lesion)/cm^2 in each segment		
			Distal	Medium-distal	Proximal-medium
1	AOM alone	7	9.83±2.75	10.70±3.56	9.44±4.66
2	AOM+500 ppm celecoxib	7	9.37±4.18 (4.7)	6.21±1.77[c] (42.0)	6.55±0.54 (30.6)
3	AOM+1500 ppm celecoxib	7	8.79±7.70 (10.6)	5.18±1.64[c] (51.6)	2.59±0.87[c,f] (72.6)
4	No treatment	7	0	0	0

CAC, β-catenin-accumulated crypts; ACF, aberrant crypt foci.
a) Mean±SD.
b–e) Significantly different from group 1 by Student's *t* test or Welch's *t* test (*b*) $P<0.004$, *c*) $P<0.02$, *d*) $P<0.002$, *e*) $P<0.001$).
f) Significantly different from group 2 by Student's *t* test ($P<0.001$).
Values in parentheses represent percentage inhibition compared to group 1.

Table IV. Mean Number of AgNORs/nucleus

Group no.	Treatment	No. of CAC (lesion) examined	Mean no. of counts in CAC	No. of ACF (lesion) examined	Mean no. of counts in ACF	No. of normal crypts examined	Mean no. of counts in normal crypts
1	AOM alone	15	3.59±0.62[a]	15	2.19±0.37	30	1.53±0.32
2	AOM+500 ppm celecoxib	15	2.43±0.39[b] (32.3)	15	2.11±0.36 (3.7)	30	1.54±0.34
3	AOM+1500 ppm celecoxib	15	2.22±0.18[b] (38.2)	15	1.79±0.37[c] (12.5)	30	1.34±0.37

CAC, β-catenin-accumulated crypts; ACF, aberrant crypt foci.
a) Mean±SD.
b) Significantly different from group 1 by Student's t test ($P<0.001$).
c) Significantly different from groups 1 and 2 by Student's t test ($P<0.03$).
Values in parentheses represent percentage decrease compared to group 1.

crypts, suggesting that β-catenin-accumulated crypts have a higher activity of cell proliferation.[19] The data for counts/nucleus in β-catenin-accumulated crypts, ACF and adjacent normal crypts are shown in Table IV. The mean AgNORs count/nucleus of β-catenin-accumulated crypts in both groups 2 and 3 was significantly smaller than that in the control group (group 1) ($P<0.001$ by Student's t test). The degree of reduction of AgNORs count/nucleus in β-catenin-accumulated crypts exceeded that seen in ACF.

DISCUSSION

Our previous studies characterizing β-catenin-accumulated crypts at the molecular and cellular levels have implicated early appearing crypts as intermediate lesions leading to colon cancer.[18, 19] β-Catenin-accumulated crypts possess characteristic morphology, and increased proliferation kinetics, and frequent mutations of *β-catenin* gene, a key gene in the process of colon cancer development in rats.[11, 23] Since inhibition of the occurrence and advancement of premalignant lesions has potential for cancer prevention, it is important to examine whether chemopreventive agents suppress the occurrence of these premalignant lesions for colorectal cancers in rats. In this report, we evaluated possible modifying effects of a selective COX-2 inhibitor, celecoxib, on the frequency and growth of β-catenin-accumulated crypts. Selection of this agent in the present study was based on previous observations by Kawamori and Reddy *et al.* that the inhibitory effects of celecoxib exceeded those of other NSAIDs,[5, 7] and the agent lacks serious side effects. It is noteworthy that celecoxib inhibited occurrence and advancement of β-catenin-accumulated crypts. These findings represent additional evidence that β-catenin-accumulated crypts are premalignant lesions of colon cancer. The results also suggest that celecoxib prevents the initial stage of colon carcinogenesis, and that the COX-2 inhibitor is a good candidate agent for prevention of colorectal carcinogenesis.

ACF are detectable in cancer-predisposed colonic mucosa with methylene blue-staining[20] and are considered to be an appropriate target for chemopreventive drug development.[24] Therefore, ACF have been used as a biomarker in studies of cancer prevention. Previous studies, including molecular analysis, have focused on the significance of ACF as early events in the colon carcinogenesis.[25] However, recent studies also reported that incidence of ACF is not always reflected in the occurrence of colon cancers.[26, 27] In the present study, celecoxib also reduced the incidence of ACF in the same manner as described previously.[21] However, the efficacy of celecoxib in reducing the frequency and growth of β-catenin-accumulated crypts was greater than that against ACF. Accordingly, the results seem to indicate that β-catenin-accumulated crypts are a novel biomarker that will be useful in studies aimed at discovering chemopreventive agents against development of colorectal cancers.

It has been shown that COX-2 inhibitor acts by its interaction with the active site of the enzyme.[28] In the present study, excess COX-2 protein was detected in cytoplasm of β-catenin-accumulated crypts. Although the exact mechanisms by which celecoxib suppresses β-catenin-accumulated crypts remains to be elucidated, accumulating evidence suggests that overexpression of COX-2 may induce growth stimulation in colon cancer cells.[29, 30] We have demonstrated that celecoxib significantly decreases proliferative activity in β-catenin-accumulated crypts. Our results may suggest that celecoxib inhibits the proliferative activity of cellular population in premalignant lesions through inhibition of the increased COX-2 activity in such early appearing lesions.

It is also interesting to note that the efficacy of celecoxib in inhibiting premalignant lesions was different in the proximal and distal colon. In this study, the suppressive efficacy in the proximal segment was greater than that in the distal colon. These findings are in agreement with the previous study showing that a COX inhibitor, piroxicam, markedly reduced the occurrence of colon tumors in the proximal colon.[31]

In conclusion, the present study indicates that chemopreventive effects of celecoxib on the initial stage of colon

carcinogenesis are related to the modulation of cell proliferation activity in putative premalignant lesions. Moreover, β-catenin-accumulated crypts may be a novel target for prevention of colorectal carcinogenesis, and could also be useful to evaluate chemopreventive agents.

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