Supplemental Table 1. Body weight, liver weight and length of large bowl of mice at week 20

Group no.	Treatment	No. of mice examined	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body weight)	Length of colon (cm)
1	AOM/DSS	15	45.1±4.1	2.4±0.3	5.2±0.6	14.1±0.9
2	AOM/DSS + 20 ppm diosgenin	15	43.1±3.3	2.6±0.4	5.9±0.7	13.7±0.8
3	AOM/DSS + 100 ppm diosgenin	15	46.8±4.8	2.6±0.3	5.5±0.4	14.1±1.0
4	AOM/DSS + 500 ppm diosgenin	15	45.3±5.6	2.6±0.3	5.8±0.8	14.3±1.0
5	AOM/DSS + 20 ppm *sanyaku*	13	44.3±4.8	2.6±0.4	5.8±0.7	13.5±1.0
6	AOM/DSS + 100 ppm *sanyaku*	15	45.9±4.7	2.5±0.3	5.4±0.7	14.2±0.8
7	AOM/DSS + 500 ppm *sanyaku*	15	45.1±3.9	2.6±0.3	5.9±0.7	14.1±0.8
8	AOM	5	42.1±2.7	2.4±0.4	5.8±0.8	14.4±0.5
9	DSS	5	43.4±2.4	2.2±0.2	5.1±0.5	13.9±1.1
10	500 ppm diosgenin	5	45.1±4.2	2.3±0.1	5.1±0.4	13.5±1.4
11	500 ppm *sanyaku*	5	42.3±2.4	2.1±0.1	5.1±0.4	14.0±0.9
12	Untreated	5	43.0±3.2	2.2±0.3	5.1±0.4	14.3±1.3

All data shown as the mean±SD.

Carcinogenesis vol.32 no.10 pp.1512–1517, 2011
doi:10.1093/carcin/bgr130
Advance Access publication July 28, 2011

Chemopreventive effects of silymarin against 1,2-dimethylhydrazine plus dextran sodium sulfate-induced inflammation-associated carcinogenicity and genotoxicity in the colon of *gpt* delta rats

Naomi Toyoda-Hokaiwado, Yumiko Yasui[1,3], Mina Muramatsu[2], Kenichi Masumura, Makiko Takamune, Masami Yamada, Toshihiro Ohta[2], Takuji Tanaka[1,4] and Takehiko Nohmi*

Division of Genetics and Mutagenesis, National Institute of Health Sciences, Tokyo 158-8501, Japan, [1]Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa 920-0293, Japan, [2]School of Life Sciences, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan, [3]Department of Physiology and Nutrition, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido 069-8501, Japan and [4]The Tokai Cytopathology Institute: Cancer Research and Prevention, Gifu 500-8285, Japan

*To whom correspondence should be addressed. Tel: +81 3 3700 9872; Fax: +81 3 3700 2348; Email: nohmi@nihs.go.jp

Silymarin, a natural flavonoid from the seeds of milk thistle, is used for chemoprevention against various cancers in clinical settings and in experimental models. To examine the chemopreventive mechanisms of silymarin against colon cancer, we investigated suppressive effects of silymarin against carcinogenicity and genotoxicity induced by 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS) in the colon of F344 *gpt* delta transgenic rats. Male *gpt* delta rats were given a single subcutaneous injection of 40 mg/kg DMH and followed by 1.5% DSS in drinking water for a week. They were fed diets containing silymarin for 4 weeks, starting 1 week before DMH injection and samples were collected at 4, 20 and 32 weeks after the DMH treatment. Silymarin at doses of 100 and 500 p.p.m. suppressed the tumor formation in a dose-dependent manner and the reduction was statistically significant. In the mutation assays, DMH plus DSS enhanced the *gpt* mutant frequency (MF) in the colon, and the silymarin treatments reduced the MFs by 20%. Silymarin also reduced the genotoxicity of DMH in a dose-dependent manner in bacterial mutation assay with *Salmonella typhimurium* YG7108, a sensitive strain to alkylating agents, and the maximum reduction was >80%. These results suggest that silymarin is chemopreventive against DMH/DSS-induced inflammation-associated colon carcinogenesis and silymarin might act as an antigenotoxic agent, in part.

Introduction

Silymarin, an extract from the milk thistle fruit (*Silybum marianum*, Family Asteraceae), has been utilized for remedy of liver diseases such as sirrhosis or hepatitis for many years (1). Silymarin is actually the collective name of the extract and composed of at least seven flavonolignans and one flavonoid, and silybinin is the major active constituent (2,3). Silymarin inhibits proliferation of various cancer cells and reduces carcinogenesis in various animal models (4,5). Therefore, it has been used in the experimental therapy of cancer and chemoprevention and even in human clinical trials. Because silymarin possesses a variety of biological properties, such as antioxidant and anti-inflammatory activities, induction of phase II enzymes and apoptosis (1), it may suppress cancer development via multiple mechanisms. However, few studies that evaluate antigenotoxic properties of silymarin are available and the contribution to the chemopreventive effects remains elusive.

In this study, we explored the antigenotoxic and chemopreventive effects of silymarin in the colon of rats. We chose colon because silymarin is highly distributed in colon mucosa when it is administered to humans orally (6) and the colon cancer is one of the most frequent human cancers worldwide (7). In fact, silymarin inhibits growth of colorectal carcinoma cells *in vitro* (8,9) and suppresses colon carcinogenesis induced by methylating agents *in vivo* (10,11). The anti-inflammatory and anticancer effects in chemically induced and spontaneous intestinal carcinogenesis in mice are also reported (12,13). To evaluate the antigenotoxic and anticarcinogenic properties, we employed F344 *gpt* delta transgenic rats treated with 1,2-dimethylhydrazine (DMH) plus dextran sodium sulfate (DSS). DMH and its metabolite azoxymethane (AOM) are potent genotoxic agents and the following treatment with a non-genotoxic agent, i.e. DSS, strongly induces inflammation in the colon, thereby enhancing colon carcinogenesis in mice (14,15) and rats (16,17). *gpt* delta transgenic rats carry approximately five copies of λEG10 DNA at a single site in the chromosome 4 (18). The λDNA carries reporter genes for *in vivo* mutagenesis, and thus point mutations and deletions can be identified in any organs of rats at the sequence levels (19,20). Because the transgene is not expressed *in vivo*, the transgenic rats are expected to display very similar sensitivity to chemical carcinogens to non-transgenic F344 rats. We also conducted bacterial mutation assay with *Salmonella typhimurium* YG7108, a sensitive strain to alkylating agents (21,22), to examine whether silymarin inhibits genotoxicity of DMH and its metabolite AOM *in vitro*. From the results, we conclude that silymarin suppresses the inflammation-associated colon carcinogenesis and suggest that the antigenotoxic property contributes to the chemopreventive effects at least partly.

Materials and methods

Materials

DMH and silymarin (silymarin group, a mixture of isomers, molecular weight = 482.44) were purchased from Sigma–Aldrich Co. (St Louis, MO). AOM, *N*-methyl-*N*-nitrosourea (MNU) and dimethyl sulfoxide were purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan).

Animals, diet and housing conditions

Male 6-week-old F344 *gpt* delta transgenic rats (20) were obtained from Japan SLC and housed three or four animals per polycarbonate cage under specific pathogen-free standard laboratory conditions: room temperature, 23 ± 2°C; relative humidity, 60 ± 5%, with a 12:12 h light–dark cycle and free access to Charles River formula-1 basal diet (Oriental Yeast Company, Tokyo, Japan) and tap water.

Treatments of animals

The protocol for this study was approved by the Animal Care and Utilization Committee of Kanazawa Medical University. One-hundred rats were randomly divided into seven groups (Figure 1). Groups 1–4 received single subcutaneous injection of DMH (40 mg/kg body wt). Groups 5–7 received no injections. One week after the carcinogen treatment, Groups 1–3 and 5 were treated with 1.5% DSS in drinking water for a week. Groups 4, 6 and 7 had just drinking water instead of 1.5% DSS solution. Groups 2, 3 and 6 were fed diets containing 100 or 500 p.p.m. silymarin for 4 weeks, starting 1 week before DMH injection. Group 7 served as an untreated control. All rats were carefully observed for clinical welfare and weighed weekly and experimental diet consumptions were recorded. Animals were killed at 4 weeks (short), 20 weeks (medium) and the experiment was terminated at 32 weeks (long).

Histological analysis

At autopsy, liver, kidneys, spleen and intestine were macroscopically examined for the presence of pathologic lesions and then isolated. The intestine was

Abbreviations: AOM, azoxymethane; DMH, 1,2-dimethylhydrazine; DSS, dextran sodium sulfate; MAM, methylazoxymethanol; MF, mutant frequency; MNU, *N*-methyl-*N*-nitrosourea.

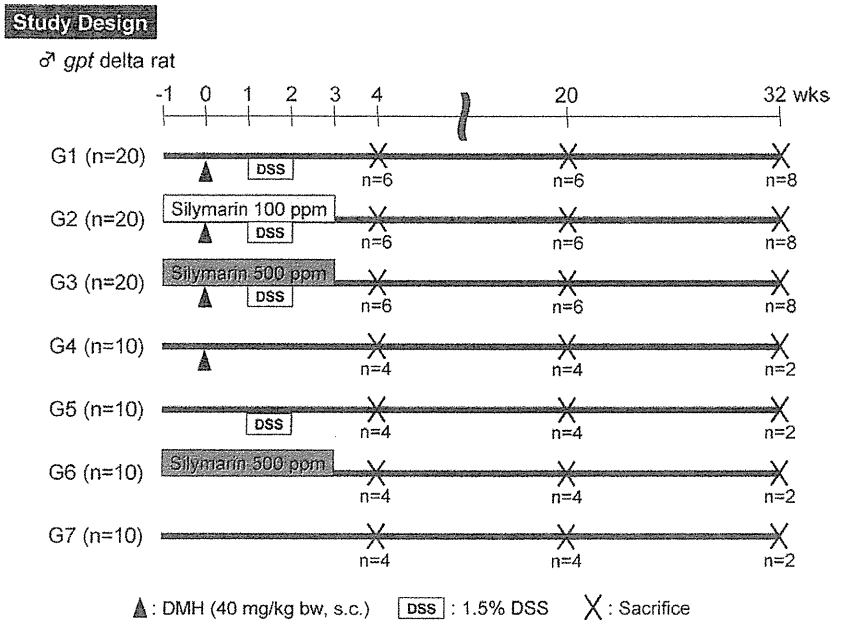

Fig. 1. Experimental protocol. Arrow heads, DMH 40 mg/kg body wt, subcutaneous injection; X, sacrifice.

excised, opened longitudinally, flushed clean with saline and examined for the presence of tumors. Colons were fixed in 10% buffered formalin and processed to hematoxylin- and eosin-stained sections. Neoplastic lesions of colorectal mucosa were histopathologically classified into dysplastic foci, adenomas and adenocarcinomas. At 4 weeks periods, 5 cm long colorectal tissues from distal segment were excised and frozen in liquid nitrogen for mutation assay. Then, colons were fixed in 10% buffered formalin and then processed for aberrant crypt foci analysis by conventional methods (23). One centimeter-long slice from stump was processed into serial paraffin sections by *en face* preparation and stained with hematoxylin and eosin and immunohistochemistry. Remained tissues were routinely embedded in paraffin and hematoxylin and eosin stained and histopathologically examined by light microscopy. The histological analysis of β-catenin-accumulated crypts and ulcer was performed based on the criteria described previously (24–26). Tumor incidence (%) means number of rat with colon tumors per total number in the experimental group and tumor multiplicity indicates number of colon tumors per rat in the experimental group.

Immunohistochemical procedures

Paraffin sections of colon were immunostained with a polyclonal anti-β-catenin antibody. Antigen retrieval was carried out by autoclaving for 15 min in 10 mmol/L citrate buffer (pH 6.0). Immunohistochemical staining was done by the avidin–biotin complex method (ABC) using the Vectastain Elite ABC system (Vector Laboratories, Burlingame, CA). Biotinylated goat anti-rabbit IgG (Vector Laboratories) was used as a secondary antibody at a dilution of 1:200. Sections were lightly counterstained with hematoxylin for microscopic examination.

DNA isolation, in vitro packaging and *gpt* mutation assay

High-molecular weight genomic DNA was extracted from the colon using the RecoverEase DNA Isolation Kit (Stratagene by Agilent Technologies, Santa Clara, CA). λEG10 phages were rescued using Transpack Packaging Extract (Stratagene). The *gpt* assay was conducted according to previously published methods (27,28). The mutant frequencies (MFs) of the *gpt* gene (*gpt* MFs) in the colon were calculated by dividing the number of confirmed 6-thioguanine (6-TG)-resistant colonies by the number of rescued plasmids. DNA sequencing of the *gpt* gene was performed with the BigDye Terminater Cycle Sequencing Ready Reaction (Applied Biosystems by Life Technologies, Carlsbad, CA) on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems). All of the confirmed *gpt* mutants recovered from the carcinogen-untreated colons and selected confirmed *gpt* mutants recovered from the carcinogen-treated colons (basically, 10 mutants per animal were analyzed) were sequenced; identical mutations from the same rat were counted as one mutant.

Bacterial reverse mutation test (Ames test)

The mutagenic activities of DMH and silymarin were assayed in a bacterial reverse mutation assay using *S. typhimurium* tester strains YG7108, as TA1535 but is $\Delta ada_{st}\Delta ogt_{st}$ (21,22). The test was conducted by the preincubation method with modification (29). Briefly, silymarin was dissolved in dimethyl sulfoxide and mixed with DMH or AOM, dissolved in distilled water. In the case of MNU, it was dissolved in dimethyl sulfoxide. The chemicals were mixed with overnight culture of YG7108 in the presence or the absence of S9 mix and incubated for 20 min at 37°C. The reaction mixture containing bacteria, an alkylating agent and silymarin was poured onto agar plates with soft agar and incubated for 2 days at 37°C. Assays were performed on triplicate.

Statistical analysis

The statistical significance of the difference in the value of MFs between treated groups and negative controls was analyzed by the Student's *t*-test. A P value <0.05 denoted the presence of a statistically significant difference. Variances in values for body weight, organ weight and pathological data were examined by Tukey multiple comparison post-test using GraghPad InStat (GraphPad Software, La Jolla, CA) to compare the differences. The tumor incidence was examined by Yates m × n χ^2-test.

Results

General conditions of animals

No marked clinical symptoms were observed during experimental periods. Body weight gain and food consumption were similar between each group. Final body weights at killing were not significantly different among groups (supplementary Tables I–III are available at *Carcinogenesis* Online).

Silymarin suppressed preneoplastic lesions

At 4 weeks necropsy, no obvious macroscopic changes were detected. Pathological findings are shown in Table I. Aberrant crypt foci developed in rats treated with DMH and DSS. The frequency of aberrant crypt foci/colon in Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500), which received DMH + DSS and silymarin at 100 and 500 p.p.m., respectively (Figure 1), was significantly lower than that of Group 1 (DMH/DSS), which received DMH + DSS alone ($P < 0.001$). The frequency of β-catenin-accumulated crypt was also reduced by dietary silymarin treatments (Group 2, $P < 0.05$; Group 3,

$P < 0.01$). In addition, number of colon mucosal ulcer was significantly reduced in these groups (Group 2, Group 3, $P < 0.001$). On microscopic observation, major changes were observed in the distal colon.

Gene mutation assay in vivo

DMH treatments enhanced *gpt* MF in the colon 100 times over the control levels (Table II). Silymarin itself was non-genotoxic [Group 6 (S500) in Figure 1]. DSS treatments did not show marked effects on the MFs. The dietary administration of silymarin at 100 and 500 p.p.m. [Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500) in Figure 1] reduced the DMH-induced MF by 20%. Because of the large standard of deviation, however, the difference was not statistically significant. Dominant base substitution induced by DMH treatment was G:C to A:T transitions and silymarin treatments did not change the mutation spectra substantially (supplementary Table IV is available at *Carcinogenesis* Online).

The colon neoplasms were reduced by silymarin treatment

The incidence and multiplicity of tumors at 20 and 32 weeks are shown in Tables III and IV, respectively. Most of adenomas and adenocarcinomas were observed in the distal colon. Although 20 week observation did not show any statistical significance between groups, the number of tumors per rat was significantly reduced by dietary silymarin administration in a dose-dependent manner at 32 weeks. In the silymarin-treated groups [Group 2 (DMH/DSS/S100) and Group 3 (DMH/DSS/S500) in Figure 1], incidence and multiplicity of proliferative lesions were markedly reduced compared with Group 1 (DMH/DSS).

Silymarin inhibited genotoxicity of DMH in S. typhimurium *YG7108*

To further characterize the suppressive effects of silymarin against DMH-induced genotoxicity, bacterial mutation assay was performed. Silymarin itself was non-genotoxic either with or without S9 activation (Figure 2 and supplementary Figure S1 is available at *Carcinogenesis* Online). DMH at a dose of 400 μg/plate, AOM at a dose of 4000 μg/plate and MNU at a dose of 10 μg/plate induced 2800, 800 and 1400 His^+ revertants per plate, respectively (Figure 2). Silymarin reduced the genotoxicity of these alkylating agents in a dose-dependent manner and the number of His^+ revertants per plate decreased by >50% at the highest dose of silymarin (Figure 2). Furthermore, silymarin showed antigenotoxic efficacy with or without S9 activation under the DMH treatments (Figure 2 and supplementary Figure S1 is available at *Carcinogenesis* Online).

Discussion

In this study, silymarin inhibited colon cancer development significantly, suggesting that it might be a quite efficient chemopreventive agent. Previously, the efficacy of silymarin against colon carcinogenesis was reported in several animal models (10–13). Kohno *et al.* (10) report that oral administration of silymarin enhances glutathione *S*-transferase activity in liver. In addition, cell proliferation in colonic mucosa is reduced and apoptosis is significantly increased by silymarin administration. Here, we reported that tumor number and incidences were greatly reduced and the MF induced by DMH was reduced by the silymarin treatments (Tables II–IV). It suggests that the antigenotoxic efficiency might contribute to the tumor reduction at least partly *in vivo*.

In *in vivo* situation, DMH is first oxidized to azomethane, which appears in the exhaled air of DMH-treated animals (30). Azomethane is oxidized to AOM, which is hydroxylated to methylazoxymethanol (MAM). AOM and MAM are also detected in the urine. MAM is unstable and decomposes to methyldiazonium, which is a highly reactive methylating intermediate (30). DMH and its metabolites, i.e. AOM and MAM, are potent carcinogens that induced colorectal carcinomas in rodent. Here, we revealed that silymarin was clearly antigenotoxic against potent alkylating carcinogens DMH, AOM and MNU *in vitro* (Figure 2). Silymarin showed similar inhibitory effects against DMH-induced genotoxicity with or without S9 mix

Table I. Pathological findings in colon (4 weeks)

Group	No. of rats	No. of mucosal ulcer/rat	No. of BCAC/rat	No. of ACF/rat	No. of foci containing	
					<4 crypts	≥4 crypts
1	6	4.50 ± 1.38[a]	3.33 ± 1.21	34.83 ± 9.20	27.17 ± 6.46	7.67 ± 3.67
2	6	1.33 ± 1.21***	1.67 ± 1.03*	17.50 ± 2.51***	16.17 ± 2.64**	1.33 ± 1.37***
3	6	0.83 ± 0.75***	0.67 ± 0.82**	13.83 ± 3.82***	13.50 ± 4.04***	0.33 ± 0.52***
4	4	0	0	0	0	0
5	4	0	0	0	0	0
6	4	0	0	0	0	0
7	4	0	0	0	0	0

BCAC, β-catenin-accumulated crypt; ACF, aberrant crypt foci.
[a]Mean ± SD.
*, **, ***: Significantly different from group 1 at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively, by Tukey multiple comparison post-test.

Table II. *gpt* MF in colon (4 weeks)

Group	Treatment			No. of rats	MF ($\times 10^{-6}$) (mean ± SD)	*P* value[a] (*t*-test)
	DMH	DSS	Test chemical			
1	+	+		6	557.7 ± 213.4 (100%)[b]	
2	+	+	Silymarin 100 p.p.m.	6	423.2 ± 246.3 (75.9%)	0.1679
3	+	+	Silymarin 500 p.p.m.	6	457.7 ± 186.5 (82.1%)	0.2039
4	+	−		4	646.8 ± 231.1 (116%)	0.2741
5	−	+		4	9.8 ± 11.0 (1.8%)	
6	−	−	Silymarin 500 p.p.m.	4	5.3 ± 5.2 (1.0%)	
7	−	−		4	5.6 ± 6.4 (1.0%)	

[a]*P* values were calculated by the Student's *t*-test.
[b]Percentage of the MF of each Group against that in Group 1.

Table III. Incidence of colon tumors in each group

Group	Treatment			Medium term (20 weeks)				Long term (32 weeks)			
	DMH	DSS	Test chemical	No. of rats	No. of rats with tumors			No. of rats	No. of rats with tumors		
					Total	AD	ADC		Total	AD	ADC
1	+	+		6	0 (0%)	0 (0%)	0 (0%)	8	8 (100%)	6 (75%)	8 (100%)
2	+	+	Silymarin 100 p.p.m.	6	1 (17%)	1 (17%)	0 (0%)	8	6 (75%)	2 (25%)	6 (75%)
3	+	+	Silymarin 500 p.p.m.	6	2 (33%)	0 (0%)	2 (33%)	8	2* (25%)	0* (0%)	2* (25%)
4	+	−		4	0	0	0	2	0	0	0
5	−	+		4	0	0	0	2	0	0	0
6	−	−	Silymarin 500 p.p.m.	4	0	0	0	2	0	0	0
7	−	−		4	0	0	0	2	0	0	0

AD, adenomas; ADC, adenocarcinomas.
*Significantly different from group 1 at $P < 0.05$ by Yates m × n χ^2-test multiple comparison post-test.

Table IV. Multiplicity of colon tumors in each group

Group	Treatment			Medium term (20 weeks)			Long term (32 weeks)		
	DMH	DSS	Test chemical	No. of tumors/rat			No. of tumors/rat		
				Total	AD	ADC	Total	AD	ADC
1	+	+		0	0	0	6.3 ± 4.8	1.6 ± 1.7	4.6 ± 3.6
2	+	+	Silymarin 100 p.p.m.	0.2 ± 0.4[a]	0.2 ± 0.4	0	1.5 ± 1.9*	0.3 ± 0.5*	1.3 ± 1.6*
3	+	+	Silymarin 500 p.p.m.	0.7 ± 1.0	0	0.7 ± 1.0	0.4 ± 0.7**	0*	0.4 ± 0.7**
4	+	−		0	0	0	0	0	0
5	−	+		0	0	0	0	0	0
6	−	−	Silymarin 500 p.p.m.	0	0	0	0	0	0
7	−	−		0	0	0	0	0	0

AD, adenomas; ADC, adenocarcinomas. Mean ± SD.
*, **: Significantly different from group 1 at $P < 0.05$ and $P < 0.01$, respectively, by Tukey multiple comparison post-test.

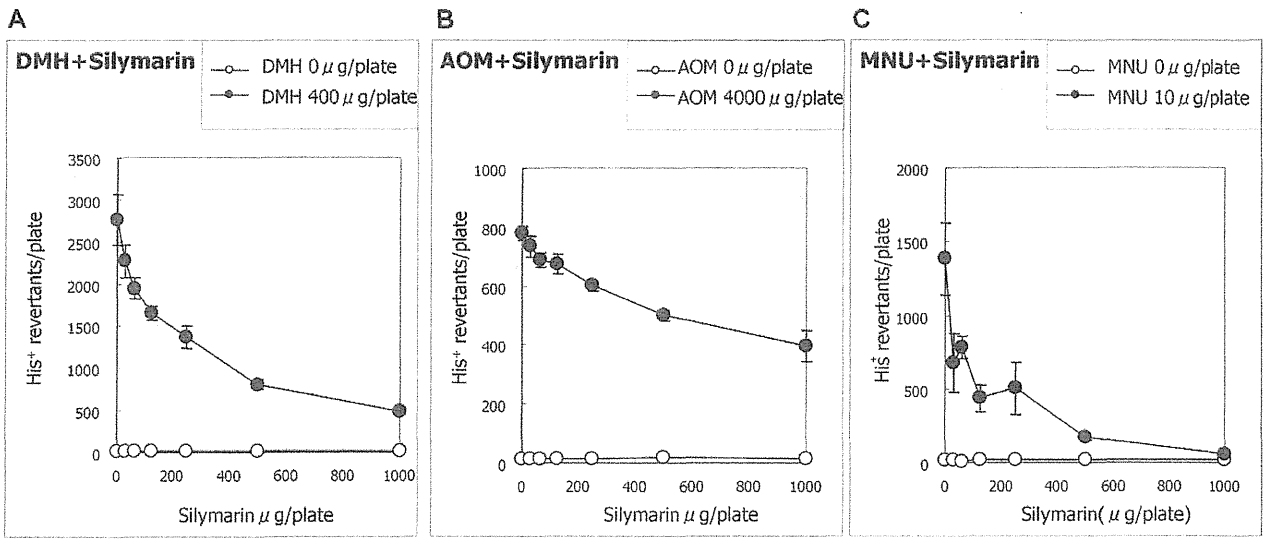

Fig. 2. Antigenotoxic activity of silymarin without S9 mix in *S. typhimurium* strain YG7108 induced by DMH (**A**), AOM (**B**) and MNU (**C**). Filled circle assayed with chemicals, open circle assayed without chemicals.

(supplementary Figure S1 is available at *Carcinogenesis* Online). It suggests that the antigenotoxic efficacy is not reduced by the metabolism *in vivo*. Nevertheless, the efficacy of antigenotoxic activity of silymarin was less pronounced *in vivo* than *in vitro*. For the bacterial mutation assays, each chemical and silymarin were directly mixed in the medium. Therefore, we speculate that the route of exposure, i.e. oral administration in rats, and the effective concentration of silymarin in the colon might account for the different efficacy between *in vivo* and *in vitro*.

When DMH is administrated by single subcutaneous injection, *N*7-methylguanine and O^6-methylguanine are detected in colon, kidney and liver in mice (31). O^6-Methylguanine DNA adduct is a potent

detrimental lesion for colorectal cancer and induces G:C to A:T transitions. The levels of O^6-methylguanine are highly distributed in the distal colon by DMH treatment (32) and histologically altered crypts often have β-catenin gene mutations (33). In the present study, the dominant base substitution in the *gpt* gene induced by DMH treatment was G:C to A:T transitions (supplementary Table IV is available at *Carcinogenesis* Online) and most of the tumors developed in distal area. To induce DNA mutations, DNA replication is required. Silymarin is known to inhibit cancer cell proliferation and induce apoptosis (8). Hence, the inhibitory effects on cell proliferation might play roles in the reduction of genotoxicity and carcinogenicity in the colon of silymarin-treated rats (Tables II–IV).

Silymarin is also reported to possess the anti-inflammatory activity (1). DMH initiation followed by DSS modification model is an established medium-term colorectal bioassay for mice (14,15) and rats (16,17). DSS induced massive inflammation on colonic mucosa by drinking administration (14,34). Under the inflammatory environment, infiltrating mast cells produced genotoxic superoxide anions. In this study, genotoxicity was not induced by DSS treatments (Table II). However, in the silymarin-treated groups, the number of colorectal mucosal tumors was reduced in a dose-dependent manner of silymarin (Tables III and IV). Oral administrated silymarin might prevent inflammation via inhibition of cytokine induction. In colon tissues from AOM-treated rats, inducible nitric oxide synthase and cyclooxygenase-2 expression levels are inhibited by dietary treatment of silibinin (35). These findings suggest that silymarin has an effective influence against promotion by DSS-induced inflammation and it might be a cause for the anticarcinogenic effect of dietary administration of silymarin.

In summary, the current study revealed the antigenotoxic potency of silymarin against alkylating agents, and suggests that the antigenotoxic efficiency along with its inhibitory effects on cell proliferation and inflammation might contribute to the effective tumor reduction *in vivo*. Our results also indicate that F344 *gpt* delta rats are useful for screening cancer chemopreventive compounds as well as environmental genotoxic carcinogens (20).

Supplementary material

Supplementary Tables I–IV and Figure S1 can be found at http://carcin.oxfordjournals.org/

Funding

This work was supported by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (MEXT, 18201010; MEXT, 22241016), the Ministry of Health, Labour and Welfare, Japan (MHLW, H21-Food-General-009) and the Japan Health Sciences Foundation (KHB1007) and for cancer research from MHLW (20 designated-8).

Conflict of Interest Statement: None declared.

References

1. Kidd,P.M. (2009) Bioavailability and activity of phytosome complexes from botanical polyphenols: the silymarin, curcumin, green tea, and grape seed extracts. *Altern. Med. Rev.*, **14**, 226–246.
2. Comelli,M.C. *et al.* (2007) Toward the definition of the mechanism of action of silymarin: activities related to cellular protection from toxic damage induced by chemotherapy. *Integr. Cancer Ther.*, **6**, 120–129.
3. Kroll,D.J. *et al.* (2007) Milk thistle nomenclature: why it matters in cancer research and pharmacokinetic studies. *Integr. Cancer Ther.*, **6**, 110–119.
4. Kaur,M. *et al.* (2007) Silymarin and epithelial cancer chemoprevention: how close we are to bedside? *Toxicol. Appl. Pharmacol.*, **224**, 350–359.
5. Ramasamy,K. *et al.* (2008) Multitargeted therapy of cancer by silymarin. *Cancer Lett.*, **269**, 352–362.
6. Hoh,C.S. *et al.* (2007) Quantitation of silibinin, a putative cancer chemopreventive agent derived from milk thistle (Silybum marianum), in human plasma by high-performance liquid chromatography and identification of possible metabolites. *J. Agric. Food Chem.*, **55**, 2532–2535.
7. Tanaka,T. (2009) Colorectal carcinogenesis: review of human and experimental animal studies. *J. Carcinog.*, **8**, 5.
8. Kaur,M. *et al.* (2009) Silibinin suppresses growth and induces apoptotic death of human colorectal carcinoma LoVo cells in culture and tumor xenograft. *Mol. Cancer Ther.*, **8**, 2366–2374.
9. Singh,R.P. *et al.* (2008) Silibinin inhibits colorectal cancer growth by inhibiting tumor cell proliferation and angiogenesis. *Cancer Res.*, **68**, 2043–2050.
10. Kohno,H. *et al.* (2002) Silymarin, a naturally occurring polyphenolic antioxidant flavonoid, inhibits azoxymethane-induced colon carcinogenesis in male F344 rats. *Int. J. Cancer*, **101**, 461–468.
11. Sangeetha,N. *et al.* (2010) Oral supplementation of silibinin prevents colon carcinogenesis in a long term preclinical model. *Eur. J. Pharmacol.*, **643**, 93–100.
12. Ravichandran,K. *et al.* (2010) Inhibitory effect of silibinin against azoxymethane-induced colon tumorigenesis in A/J mice. *Clin. Cancer Res.*, **16**, 4595–4606.
13. Rajamanickam,S. *et al.* (2010) Chemoprevention of intestinal tumorigenesis in APCmin/+ mice by silibinin. *Cancer Res.*, **70**, 2368–2378.
14. Tanaka,T. *et al.* (2003) A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate. *Cancer Sci.*, **94**, 965–973.
15. Kohno,H. *et al.* (2005) Beta-catenin mutations in a mouse model of inflammation-related colon carcinogenesis induced by 1,2-dimethylhydrazine and dextran sodium sulfate. *Cancer Sci.*, **96**, 69–76.
16. Tanaka,T. *et al.* (2009) Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats. *Chem. Biol. Interact.*, **177**, 128–136.
17. Cho,Y.M. *et al.* (2008) A new medium-term rat colorectal bioassay applying neoplastic lesions as end points for detection of carcinogenesis modifiers effects with weak or controversial modifiers. *Toxicol. Pathol.*, **36**, 459–464.
18. Hayashi,H. *et al.* (2003) Novel transgenic rat for *in vivo* genotoxicity assays using 6-thioguanine and Spi$^-$ selection. *Environ. Mol. Mutagen.*, **41**, 253–259.
19. Nohmi,T. *et al.* (2005) Molecular nature of intrachromosomal deletions and base substitutions induced by environmental mutagens. *Environ.Mol. Mutagen.*, **45**, 150–161.
20. Toyoda-Hokaiwado,N. *et al.* (2010) Integration of *in vivo* genotoxicity and short-term carcinogenicity assays using F344 *gpt* delta transgenic rats: *in vivo* mutagenicity of 2,4-diaminotoluene and 2,6-diaminotoluene structural isomers. *Toxicol Sci.*, **114**, 71–78.
21. Yamada,M. *et al.* (1997) New tester strains of *Salmonella typhimurium* lacking O^6-methylguanine DNA methyltransferases and highly sensitive to mutagenic alkylating agents. *Mutat. Res.*, **381**, 15–24.
22. Yamada,M. *et al.* (1995) Construction and characterization of mutants of *Salmonella typhimurium* deficient in DNA repair of O^6-methylguanine. *J. Bacteriol.*, **177**, 1511–1519.
23. Bird,R.P. (1987) Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: preliminary findings. *Cancer Lett.*, **37**, 147–151.
24. Yamada,Y. *et al.* (2001) Sequential analysis of morphological and biological properties of beta-catenin-accumulated crypts, provable premalignant lesions independent of aberrant crypt foci in rat colon carcinogenesis. *Cancer Res.*, **61**, 1874–1878.
25. Cooper,H.S. *et al.* (1993) Clinicopathologic study of dextran sulfate sodium experimental murine colitis. *Lab Invest*, **69**, 238–249.
26. Suzuki,R. *et al.* (2004) Sequential observations on the occurrence of preneoplastic and neoplastic lesions in mouse colon treated with azoxymethane and dextran sodium sulfate. *Cancer Sci.*, **95**, 721–727.
27. Nohmi,T. *et al.* (1996) A new transgenic mouse mutagenesis test system using Spi$^-$ and 6-thioguanine selections. *Environ. Mol. Mutagen.*, **28**, 465–470.
28. Nohmi,T. *et al.* (2000) Recent advances in the protocols of transgenic mouse mutation assays. *Mutat. Res.*, **455**, 191–215.
29. Maron,D.M. *et al.* (1983) Revised methods for the Salmonella mutagenicity test. *Mutat. Res.*, **113**, 173–215.
30. Fiala,E.S. (1977) Investigations into the metabolism and mode of action of the colon carcinogens 1,2-dimethylhydrazine and azoxymethane. *Cancer*, **40**, 2436–2445.
31. Cooper,H.K. *et al.* (1978) DNA alkylation in mice with genetically different susceptibility to 1,2-dimethylhydrazine-induced colon carcinogenesis. *Cancer Res.*, **38**, 3063–3065.

32. Jackson,P.E. *et al.* (2003) Associations between tissue-specific DNA alkylation, DNA repair and cell proliferation in the colon and colon tumour yield in mice treated with 1,2-dimethylhydrazine. *Carcinogenesis*, **24**, 527–533.
33. Yamada,Y. *et al.* (2000) Frequent beta-catenin gene mutations and accumulations of the protein in the putative preneoplastic lesions lacking macroscopic aberrant crypt foci appearance, in rat colon carcinogenesis. *Cancer Res.*, **60**, 3323–3327.
34. Suzuki,R. *et al.* (2005) Dose-dependent promoting effect of dextran sodium sulfate on mouse colon carcinogenesis initiated with azoxymethane. *Histol. Histopathol.*, **20**, 483–492.
35. Velmurugan,B. *et al.* (2008) Inhibition of azoxymethane-induced colonic aberrant crypt foci formation by silibinin in male Fisher 344 rats. *Cancer Prev. Res. (Phila)*, **1**, 376–384.

Received February 13, 2011; revised June 9, 2011; accepted July 29, 2011

Chemico-Biological Interactions 193 (2011) 79–87

Contents lists available at ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice via modulation of the inflammatory cytokines

Yumiko Yasui [a,b,*], Masashi Hosokawa [c], Nana Mikami [c], Kazuo Miyashita [c], Takuji Tanaka [b,d]

[a] School of Veterinary Medicine, Rakuno Gakuen University, 069-8501 Hokkaido, Japan
[b] Department of Oncologic Pathology, Kanazawa Medical University, 920-0293 Ishikawa, Japan
[c] Faculty of Fisheries Sciences, Hokkaido University, 041-8611 Hokkaido, Japan
[d] The Tokai Cytopathology Institute, Cancer Research and Prevention, 500-8285 Gifu, Japan

ARTICLE INFO

Article history:
Received 14 March 2011
Received in revised form 12 May 2011
Accepted 13 May 2011
Available online 20 May 2011

Keywords:
Astaxanthin
Chemoprevention
Colorectal carcinogenesis
Inflammation
Mouse

ABSTRACT

Astaxanthin (AX) is one of the marine carotenoid pigments, which possess powerful biological antioxidant, anti-inflammatory and anti-cancer properties. The purpose of this study is to investigate possible inhibitory effect of AX against inflammation-related mouse colon carcinogenesis and dextran sulfate sodium (DSS)-induced colitis in male ICR mice. We conducted two different experiments. In the first experiment, we evaluated the effects of AX at three dose levels, 50, 100 and 200 ppm in diet, on colitis-associated colon carcinogenesis induced by azoxymethane (AOM)/DSS in mice. In the second, the effects of the AX (100 and 200 ppm) in diet on DSS-induced colitis were determined. We found that dietary AX significantly inhibited the occurrence of colonic mucosal ulcers, dysplastic crypts, and colonic adenocarcinoma at week 20. AX-feeding suppressed expression of inflammatory cytokines, including nuclear factor (NF)-κB, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, inhibited proliferation, and induced apoptosis in the colonic adenocarcinomas. Feeding with 200 ppm AX, but not 100 ppm, significantly inhibited the development of DSS-induced colitis. AX feeding (200 ppm in diet) also lowered the protein expression of NF-κB, and the mRNA expression of inflammatory cytokines, including IL-1β, IL-6, and cyclooxygenase (COX)-2. Our results suggest that the dietary AX suppresses the colitis and colitis-related colon carcinogenesis in mice, partly through inhibition of the expression of inflammatory cytokine and proliferation. Our findings suggest that AX is one of the candidates for prevention of colitis and inflammation-associated colon carcinogenesis in humans.

1. Introduction

Astaxanthin (AX) is a red-orange colored xanthophyll carotenoid with powerful biological antioxidant. This naturally occurred carotenoid is present in salmonid, shrimp, and crustacean aquaculture to provide the pink color characteristic of that species. *Haematococcus pluvialis* is the richest source of natural AX and is able to now cultivate at industrial scale. AX, similar to other carotenoids, cannot be synthesized by animals and must be provided in the diet. Various biological activities of AX have been reported. They include anti-oxidant, anti-inflammatory and anti-cancer properties [1–4]. Increasing evidence suggests that AX is a potent anti-tumor agent in several experimental animal models. For example, AX suppresses urinary bladder carcinogenesis in mice [5] and colon carcinogenesis in rats [6] and oral carcinogenesis in rats [2]. Recent studies have demonstrated that dietary administration with AX inhibits the early phase of 1,2-dimethylhydrazine (DMH)-induced colon carcinogenesis in rat [7,8].

Epidemiological studies have revealed an association between nutritional habits and the prevention of certain types of cancer, including colorectal cancer (CRC). CRC is the second commonest malignancy in the developed countries and significant cause of mortality. Chemoprevention uses specific natural or synthetic compounds in an attempt to prevent, halt or reverse the process of carcinogenesis, and is an attractive way to fight CRC development [9–11]. Recently, considerable attention has been focused on identification of phytochemicals, particularly those included in our diet, which has the ability to interfere with carcinogenic or mutagenic process [12–14].

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; AX, astaxanthin; COX-2, cyclooxygenase-2; CRC, colorectal cancer; DMH, 1,2-dimethylhydrazine; DSS, dextran sulfate sodium; H&E, hematoxylin and eosin; IAPs, inhibitor proteins of apoptosis; ICR, Crj: CD-1; IL-1β, interleukin-1β; iNOS, nitric oxide synthase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PCNA, proliferating cell nuclear antigen; TNF-α, tumor necrosis factor-α; UC, ulcerative colitis.

* Corresponding author at: School of Veterinary Medicine, Rakuno Gakuen University, 582 Midorimachi, Bunkyodai, Ebetsu, Hokkaido 069-8501, Japan. Tel.: +81 11 388 4854; fax: +81 11 387 5890.
E-mail address: y-yasui@rakuno.ac.jp (Y. Yasui).

0009-2797/$ - see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.cbi.2011.05.006

In human colorectal carcinogenesis, patients with ulcerative colitis (UC) as well as those with Crohn's disease are at an increased risk for the development of CRC. Unlike sporadic CRC, CRC in UC patients arises from a focal or multifocal dysplastic mucosa in areas of inflammation. Using our novel colitis-related and two-stage mouse CRC model that mimics human CRC in inflamed colon [15], we have reported several synthetic and natural compounds which effectively suppressed colitis-associated colon carcinogenesis [16–20]. In this model, several colon carcinogens, including azoxymethane (AOM) are used as an initiator at a low dose and a colitis-inducing agent dextran sulfate sodium (DSS) is used as a tumor-promoter. The regimens can be applied to rats to induce CRC within a short-term period [18].

The nuclear factor (NF)-κB is a well established regulator of genes encoding cytokines, cytokine receptors and cell adhesion molecules that drive immune and inflammatory responses [21]. Recently, NF-κB activation has also been connected with multiple aspects of oncogenesis [22]. Expression of several genes, such as cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)-α, interleukin (IL)-1β, cell surface adhesion molecules and anti-apoptotic proteins which are involved in tumor initiation, tumor promotion and metastasis are regulated by NF-κB [23]. Therefore, NF-κB has become one of the most important targets of cancer chemoprevention.

This study was aimed to investigate possible inhibitory effect of AX against the colitis-associated colon carcinogenesis using our AOM/DSS mouse model. The study contained two different experiments. In the first experiment, we evaluated the effects of AX at three dose levels, 50, 100 and 200 ppm in diet, on colitis-associated colon carcinogenesis in mice. Also, immunohistochemical expressions of NF-κB, a proliferation marker of proliferating cell nuclear antigen (PCNA), an apoptosis marker of survivin, and certain inflammatory cytokines, including TNF-α and IL-1β, in colonic adenocarcinomas were analyzed, since inflammatory cytokines and proliferation activity play a significant role in inflammation-associated carcinogenesis [24,25]. In the second, we determined the effects of the AX at two doses, 100 and 200 ppm in diet, on colonic inflammation induced by DSS and expression of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, COX-2, and iNOS in mice.

2. Material and methods

2.1. Animals, chemicals, and diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Inc., Tokyo) aged 5 weeks were used in this study. They were maintained at Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guideline. All animals were housed in plastic cages (4 or 5 mice/cage) with free access to drinking water and a basal diet, AIN-76A, under controlled conditions of humidity (50 ± 10%), light (12/12 h light/dark cycle) and temperature (23 ± 2 °C). After arrival, the animals were quarantined for the first 7 days, and then randomized by body weights into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma Chemical Co. (St. Louis, MO). DSS with a molecular weight of 36,000–50,000 (Cat. No. 160110) was purchased from MP Biomedicals, LLC (Aurora, OH). For the induction of colitis, DSS was dissolved in water at a concentration of 1.5% (w/v). The experiments were approved by Kanazawa Medical University. AX was purchased from Sigma–Aldrich, Inc. (Tokyo, Japan).

2.2. Experiment 1

A total of 150 male ICR mice were divided into 6 experimental and control groups (Fig. 1a). Mice in groups 1–4 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, animals received 1.5% DSS in the drinking water for 7 days. Subsequently, they received the diets containing 0, 50, 100, and 200 ppm AX for 17 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 5 was not treated with AOM and DSS, and was fed the diet containing 200 ppm AX. Group 6 was served as an untreated control. Animals were sequentially sacrificed at weeks 5 and 20 by ether overdose to determine the effects of AX on colon tumorigenesis and expression of certain inflammatory cytokines. At sacrifice, complete necropsies were done on all mice. After macroscopic inspection, tissues from large bowel, liver, and kidney were processed to histological examination by conventional methods as did in experiment 1. Histopathological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining.

2.3. Experiment 2

A total of 60 male ICR mice were divided into 4 experimental and control groups and subjected to 5-week experiment (Fig. 1b). Mice in groups 1–3 were fed the experimental diets containing 0, 100, and 200 ppm AX, respectively, during the entire experimental period. Four weeks after the start of the experiment, mice in groups 1 through 3 were given 1.5% DSS in their drinking water for 7 days. Group 4 was served as an untreated control. All animals were sacrificed at week 5. At sacrifice, body, liver, and kidney were weighted, and then liver and kidney were fixed in 10% buffered formalin for 24 h. The large bowels were flushed with saline, and excised. After measurement of their length (from the ileocecal junction to the anal verge), they were cut open longitudinally along the main axis, and gently washed with saline. The large bowel was macroscopically inspected for the presence of pathological lesions, including ulceration, cut, and fixed in 10% buffered formalin for 24 h. A histopathological examination was performed on paraffin-embedded sections from large bowel after H&E staining to determine inflammation score in the colonic mucosa.

2.4. Scoring of inflammation in the large bowel

Inflammation in the large bowel was scored on the H&E-stained sections. Large intestinal inflammation was graded according to the morphological criteria described by Cooper et al. [26]: grade 0, normal appearance; grade 1, shortening and loss of the basal 1/3 of the actual crypts with mild inflammation in the mucosa; grade 2, loss of the basal 2/3 of the crypts with moderate inflammation in the mucosa; grade 3, loss of all of the crypts with severe inflammation in the mucosa and submucosa, while retaining the surface epithelium and grade 4, presence of mucosal ulcer with severe inflammation (infiltration of neutrophils, lymphocytes, and plasma cells) in the mucosa, submucosa, muscularis propria and/or subserosa. The scoring was performed on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

2.5. Immunohistochemistry

For immunohistochemistry using the labeled streptavidin biotin method, with a LSAB Kit (DAKO Japan, Kyoto, Japan), with microwave accentuation, 4-μm-thick paraffin-embedded sections from the colons of mice in all groups of two experiments were made. The paraffin-embedded sections were heated for 30 min at 65 °C, deparaffinized in xylene and rehydrated through graded ethanol solutions at room temperature. A 0.05 M Tris HCl buffer (pH 7.6) was used to prepare solutions to rinse slides between the various steps. Incubations were performed in a humidified chamber. The sections were treated for 40 min at room temperature with

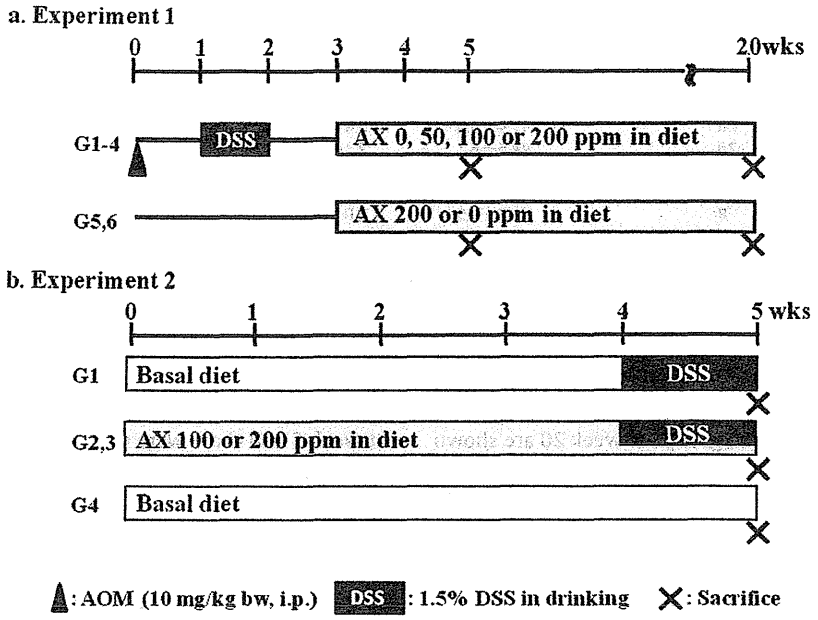

Fig. 1. Experimental protocols for two different studies. In the first experiment, all mice were divided into the following six experimental and control groups. Mice in G1–G4 were given a single intraperitoneal injection of AOM and received 1.5% DSS in the drinking water. Mice in G2–G4 were fed the experimental diets containing 50, 100, and 200 ppm of AX, respectively, for the entire 17-week experimental period. G5 was given 200 ppm AX alone for 17 weeks. G6 served as an untreated control. In the second experiment, all mice were divided into the four groups. Mice in G1 was treated with 1.5% DSS alone for 7 days before the end of the study. G2 and G3 were fed the experimental diets containing 100 and 200 ppm AX, respectively, for the entire 5-week experimental period. G4 served as an untreated control.

2% bovine serum albumin and incubated overnight at 4 °C with primary antibodies. Primary antibodies used were anti-human TNF-α rabbit polyclonal antibody (#ab6671, 1:500 dilution; Abcam, Inc., Cambridge, MA), anti-human PCNA mouse monoclonal antibody (DAKO #U 7032, 1:1000 dilution; DakoCytomation, Kyoto, Japan), anti-human survivin rabbit monoclonal antibody (71G4B7, #2808, 1:400 dilution, Cell Signaling Technology Japan, Tokyo, Japan), anti-NF-κB p50 (H-119) rabbit polyclonal antibody (#sc-7178, 1:500 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), and anti-mouse IL-1β rabbit polyclonal antibody (#LS-B40, 1:250 dilution; LifeSpan BioSciences, Inc., Seattle, WA). These antibodies were applied to the sections according to the manufacturer's protocol. The horseradish peroxidase activity was visualized by the treatment with H_2O_2 and 3,3′-diaminobenzidine for 5 min. At the last step, the sections were weakly counterstained with Mayer's hematoxylin (Merck, Tokyo, Japan). For each case, the negative controls were performed on the serial sections without the first antibodies.

2.6. Immunohistochemical evaluation and scoring

Immunohistochemical analysis was done in 6 mice from group 1 and 5 mice each from groups 2–4. The immunoreactivity against the antibodies, except PCNA, and survivin, was assessed in the large colonic adenocarcinomas (more than 3 mm in diameter) developed in groups 1–4 using a microscope (Olympus BX41, Olympus Optical Co., Tokyo, Japan). The intensity and localization of the immunoreactivity against the primary antibodies were determined by a pathologist (T.T.) who was unaware of the treatment group to which the slide belonged. The number of nuclei with positive reactivity for PCNA-, and survivin-immunohistochemistry were counted in a total of 3 × 100 cells in three different areas of the colonic cancer and expressed as a percentage (mean ± SD). The immunoreactivity was evaluated against the TNF-α, IL-1β and NF-κB antibodies with grading between 0 and 5: 0 (~15% of the colonic cancer cells showing positive reactivity), 1 (16–30% of the colonic cancer cells showing positive reactivity), 2 (31–45% of the colonic cancer cells showing positive reactivity), 3 (46–60% of the colonic cancer cells presenting positive reactivity), 4 (61–75% of the colonic cancer cells showing positive reactivity), and 5 (75%~ of the colonic cancer cells showing positive reactivity).

2.7. Total RNA extraction and quantitative real time PCR

Total RNA was extracted from colonic mucosa using the RNeasy Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer's protocol. The cDNA was then synthesized from total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems Japan Ltd., Tokyo, Japan). Quantitative real time PCR analysis of individual cDNA was performed with ABI Prism 7500 (Applied Biosystems Japan Ltd., Tokyo, Japan) using TaqMan Gene Expression Assays (Applied Biosystems Japan Ltd., Tokyo, Japan; TNF-α: Mm00443258_m1, IL-6: Mm00446190_m1, COX-2: Mm00478374_m1, iNOS: Mm00440485_m1, and β-actin: Mm00607939_s1). PCR cycling conditions were 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

2.8. Statistical analysis

All measurements were statistically analyzed using either the Tukey multiple comparison post test or Fisher's extract probability test. Differences were considered to be statistically significant at $P < 0.05$.

3. Results

3.1. Experiment 1

3.1.1. General observation

Feeding with three dose levels of AX-containing diets did not produce any observable toxicity or any gross change in any organ

Table 1
Body, liver weight and length of large bowel of mice at week 20 (Experiment 1).

Group no.	Treatment	No. of mice examined	Body wt (BW, g)	Liver wt (g)	Relative liver wt (g/100g BW)	Length of large bowel (cm)
1	AOM/DSS	25	42.4 ± 5.0[a]	2.2 ± 0.4	5.1 ± 0.7	12.9 ± 1.7
2	AOM/DSS/50 ppm AX	20	47.4 ± 6.6[b]	2.4 ± 0.5	5.1 ± 0.5	12.5 ± 1.2
3	AOM/DSS/100 ppm AX	20	44.6 ± 6.1	2.3 ± 0.4	5.3 ± 0.8	12.4 ± 1.2
4	AOM/DSS/200 ppm AX	20	41.9 ± 4.9	2.0 ± 0.4	4.8 ± 0.7	12.2 ± 0.9
5	200 ppm AX	8	43.3 ± 4.1	2.0 ± 0.3	4.7 ± 0.7	12.2 ± 1.5
6	None	8	41.7 ± 4.5	2.0 ± 0.2	4.7 ± 0.3	12.4 ± 0.9

[a] Mean ± SD.
[b] $P < 0.05$ vs. group 1.

examined. This was confirmed by histopathological examinations in liver and kidney of the mice. Histology revealed no morphological evidence of fatty liver. The mean weights of body, liver (g/100 g body weight) and colon length in all groups at week 20 are shown in Table 1. The mean body weight of group 2 was significantly increased compared with group 1 ($P < 0.05$). Other measures did not significantly differ among the groups.

3.1.2. Incidence and multiplicity of colonic mucosal ulcer and dysplasia

The incidences and multiplicity of colonic mucosal ulcer and colonic dysplasia at weeks 5 and 20 were listed in Table 2. The incidence of mucosal ulcer was decreased with increasing the dose of AX at week 20. The decrease in mice fed the diet containing 200 ppm AX was statistically significant ($P < 0.05$). Similarly, administration with 100 and 200 ppm AX significantly decreased the incidence of colonic dysplasia at week 20 ($P < 0.05$), but the doses did not significantly affect the incidence at week 5. In the multiplicity of colonic lesions, the both values were reduced by the treatment with AX, but the statistically significant differences were not obtained among the groups at week 5. However, at week 20, dietary administration with AX at three doses significantly reduced the multiplicity of ulcer and dysplasia (mucosal ulcer: $P < 0.01$ at 50 and 100 ppm AX, $P < 0.001$ at 200 ppm AX; dysplasia: $P < 0.05$ at 50 ppm AX, $P < 0.01$ at 100 and 200 ppm AX).

3.1.3. Incidence and multiplicity of colonic adenoma and adenocarcinoma

The incidences and multiplicity of colonic tumor at weeks 5 and 20 were listed in Table 3. At week 5, a few mice of group 1 (AOM + DSS) and 4 (AOM + DSS + 200 ppm AX) developed a few colonic tumors. At week 20, group 1 had colonic adenocarcinoma with the incidence of 92%. Treatment with all three doses of AX significantly reduced the incidence of adenocarcinoma ($P < 0.01$). As to the incidence of colonic adenoma, dietary AX decreased the value, but the differences were insignificant. The multiplicities of colonic adenoma and adenocarcinoma of groups 1 and 4 were small at week 5 and there were no colonic tumors in the mice of groups 2, 3, 5, and 6. At week 20, dietary administration with AX lowered the multiplicities of colonic adenoma and adenocarcinoma and treatment with 200 ppm AX significantly reduced the multiplicity of adenocarcinoma ($P < 0.05$).

3.1.4. Cell proliferation and apoptosis

We immunohistochemically analyzed the expression of PCNA and survivin of colonic adenocarcinomas to examine the effect of AX on cell proliferation and apoptosis at week 20. As shown in Fig. 2a, the mean PCNA-labeling indices of colonic adenocarcinomas in groups 3 ($P < 0.01$) and 4 ($P < 0.001$) were significantly smaller than that of group 1. The surviving-positive indices of groups 2 ($P < 0.05$), 3 ($P < 0.001$) and 4 ($P < 0.001$) were significantly low when compared to that of group 1 (Fig. 2b).

3.1.5. Immunohistochemical expression of inflammatory cytokines

Fig. 3 shows the immunohistochemical expression level of TNF-α, IL-1β, and NF-κB of adenocarcinomas at week 20. Dietary AX suppressed the expression of all the three cytokines. Administration with 200 ppm AX significantly reduced the expression revel of TNF-α ($P < 0.01$), IL-1β ($P < 0.05$) and NF-κB ($P < 0.01$) compared with group 1.

3.2. Experiment 2

3.2.1. General observation

Feeding with experimental diets containing two dose levels of AX did not produce any observable toxicity or any gross change in any organ examined. This was confirmed by histopathological examinations in liver and kidney of the mice. Histology revealed

Table 2
Incidence and multiplicity of colonic lesions in each group at weeks 5 and 20 (Experiment 1).

Group no.	Treatment	Mucosal ulcer (incidence/multiplicity)		Dysplasia (incidence/multiplicity)	
		Week 5	Week 20	Week 5	Week 20
1	AOM/DSS	4/5, 80% 2.4 ± 1.5[a]	13/17, 77% 2.4 ± 1.8	4/5, 80% 4.2 ± 3.4	15/17, 88% 1.8 ± 1.1
2	AOM/DSS/50 ppm AX	3/5, 60% 1.4 ± 1.3	6/12, 50% 0.7 ± 0.8[b]	2/5, 40% 0.8 ± 1.1	7/12, 58% 0.9 ± 0.9 [c]
3	AOM/DSS/100 ppm AX	4/5, 80% 1.4 ± 1.1	5/12, 42% 0.7 ± 0.9[b]	2/5, 40% 0.6 ± 0.9	6/12, 50%[c] 0.6 ± 0.7[b]
4	AOM/DSS/200 ppm AX	4/5, 80% 1.2 ± 1.1	4/12, 33%[c] 0.4±0.7[d]	4/5, 80% 1.8±1.6	5/12, 42%[c] 0.5 ± 0.7[b]
5	200 ppm AX	0/3, 0% 0	0/2, 0% 0	0/3, 0% 0	0/2, 0% 0
6	None	0/3, 0% 0	0/2, 0% 0	0/3, 0% 0	0/2, 0% 0

[a] Mean ± SD.
[b,c,d] Significantly different from group 1 at $P < 0.01$, $P < 0.05$ and $P < 0.001$, respectively.

Table 3
Incidence and multiplicity of colonic tumors in each group at weeks 5 and 20 (Experiment 1).

Group no.	Treatment	Adenoma (incidence/multiplicity)		Adenocarcinoma (incidence/multiplicity)		Total (incidence/multiplicity)	
		Week 5	Week 20	Week 5	Week 20	Week 5	Week 20
1	AOM/DSS	2/5, 40% 0.8±1.1 [a]	7/12, 58% 1.3±1.4	1/5, 20% 0.2±0.4	11/12, 92% 1.7±1.0	2/5, 40% 1.0±1.4	12/12, 100% 3.0±2.9
2	AOM/DSS/50 ppm AX	0/5, 0% 0	4/12, 33% 0.9 ± 1.6	0/5, 0% 0	4/12, 33% [b] 0.6±1.2	0/5, 0% 0	6/12, 50% [c] 1.5 ± 2.6
3	AOM/DSS/100 ppm AX	0/5, 0% 0	5/12, 42% 0.7 ± 1.0	0/5, 0% 0	4/12, 33% [b] 0.7±1.1	0/5, 0% 0	6/12, 50% [c] 1.3±2.0
4	AOM/DSS/200 ppm AX	1/5, 20% 0.4 ± 0.9	6/12, 50% 0.9 ± 1.3	1/5, 20% 0.8±1.8	4/12, 33% [b] 0.5±0.8[c]	2/5, 40% 1.2±1.8	7/12, 58% [c] 1.4±1.8
5	200 ppm AX	0/3, 0% 0	0/2, 0% 0	0/3, 0% 0	0/2, 0% 0	0/3, 0% 0	0/2, 0% 0
6	None	0/3, 0% 0	0/2, 0% 0	0/3, 0% 0	0/2, 0% 0	0/3, 0% 0	0/2, 0% 0

[a] Mean ± SD.
[b,c] Significantly different from group 1 at $P < 0.01$ and $P < 0.05$, respectively.

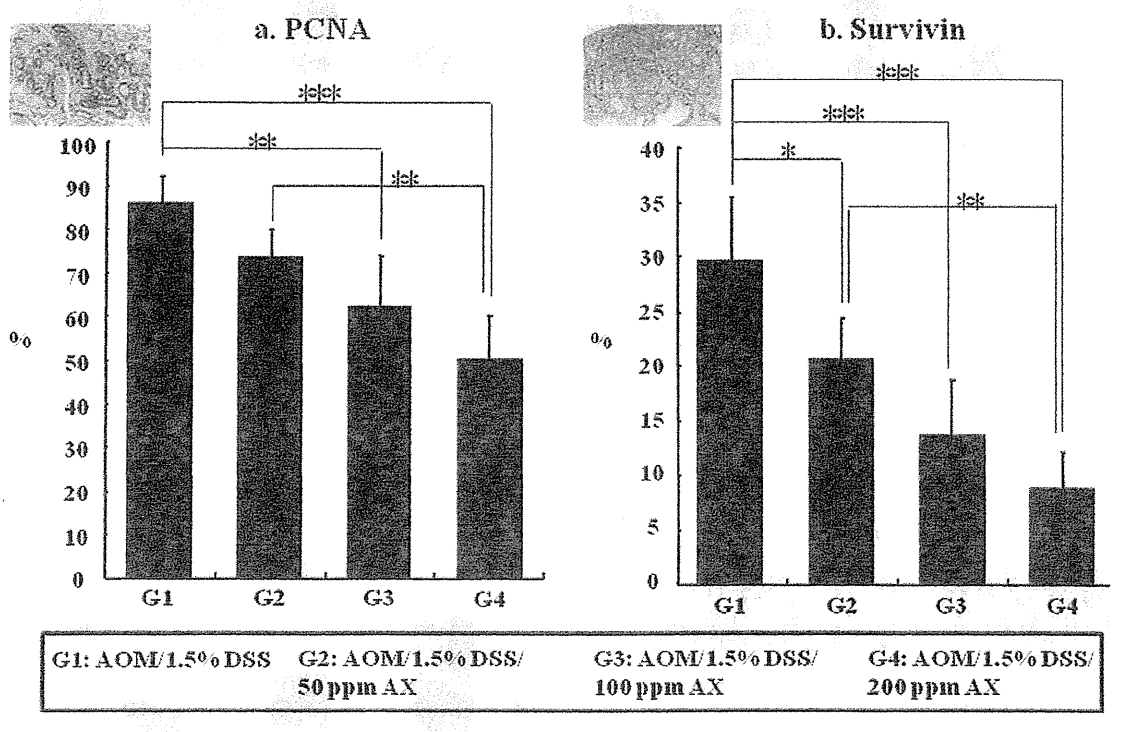

Fig. 2. Indices of PCNA-labeled nuclei and survivin in adenocarcinomas developed in the colon of a mouse from groups 1–4 (G1; $n = 6$, G2–4; $n = 5$). *, **, ***: Significantly different at $P < 0.05$, $P < 0.01$, and, $P < 0.001$, respectively, by Tukey multiple comparison post test.

no morphological evidence of fatty liver. The mean weights of body, liver, relative liver (g/100 g body weight) and colon length in all groups at sacrifice are shown in Table 4. Although, it was observed that length of large bowel of mice in Group 1–3 treated with DSS were shorter than untreated mice, there were no significant differences of the any date among the groups.

3.2.2. Inflammation score in the large bowel

Fig. 4 shows the histochemical staining of colonic mucosa (Fig. 4a–d), and summarized the scoring of colonic inflammation (Fig. 4e) at week 5. The inflammation scores of groups 1 (1.5% DSS alone, Fig. 4a), 2 (1.5% DSS + 100 ppm AX, Fig. 4b), and 3 (1.5% DSS + 200 ppm AX, Fig. 4c) were significantly greater than that of group 4 (untreated, Fig. 4d). Dietary AX dose-dependently decreased the colonic inflammation. In particular, feeding with 200 ppm AX (group 3) significantly decreased the colonic inflammation score when compared to group 1 ($P < 0.05$).

3.2.3. Expression of inflammatory cytokine in colonic mucosa

Fig. 5 showed the relative mRNA expression level of TNF-α, IL-1β, IL-6, COX-2, and iNOS when the value of group 1 was converted to 100 by RT-PCR analysis. The expression of all inflammatory cytokines in the mice treated with DSS was increased in comparison with untreated mice. The expression of IL-1β, IL-6, and COX-2 were suppressed by administrated with AX dose-dependently. When fed the diet containing 100 ppm AX, the expression of IL-1β, IL-6, and COX-2 decreased to 40%, 30%, and 50% of group 1, respectively. Furthermore, the treatment with 200 ppm AX caused 80% reduction in the expression of IL-1β, IL-6, and COX-2 when compared to the DSS alone group. As to iNOS expression, 100 ppm AX increased the expression, but 200 ppm AX feeding decreased the expression.

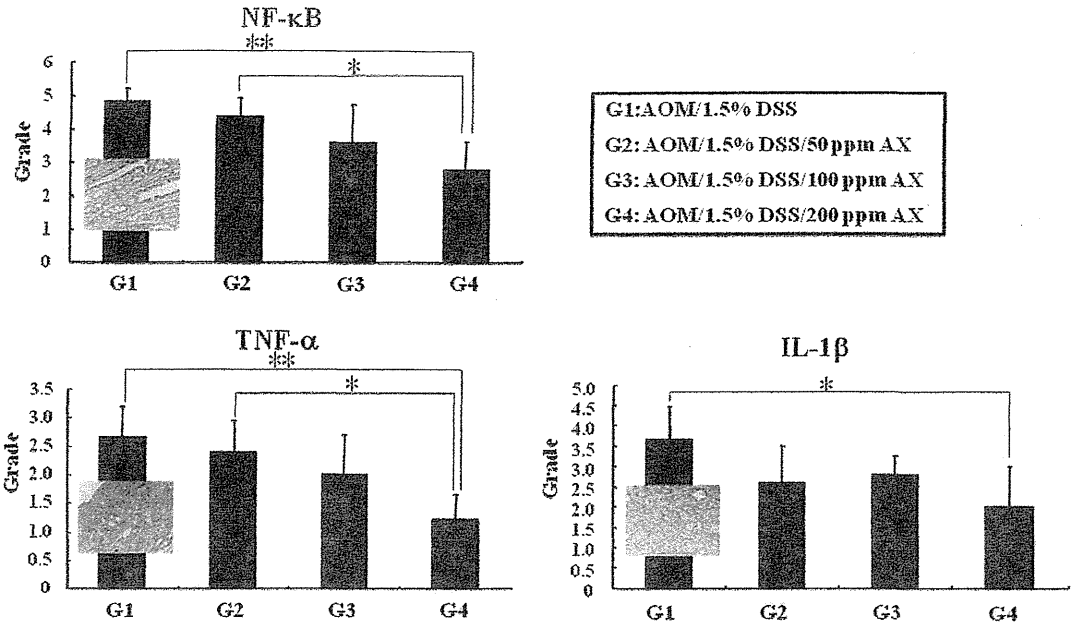

Fig. 3. Immunohistochemical analysis of NF-κB, TNF-α, and IL-1β in the colon tumor of a mouse from groups 1–4 (G1; $n = 6$, G2–4; $n = 5$). *, **: Significantly different at $P < 0.05$ and $P < 0.01$, respectively, by Tukey multiple comparison post test.

Table 4
Body, liver weight and length of large bowel of mice at week 5 (Experiment 2).

Group no.	Treatment	No. of mice examined	Body wt (BW, g)	Liver wt (g)	Relative liver wt (g/100 g BW)	Length of large bowel (cm)
1	1.5% DSS	15	38.4 ± 2.3[a]	2.2 ± 0.2	5.7 ± 0.4	11.4 ± 1.0
2	100 ppm AX/1.5% DSS	15	37.7 ± 4.2	2.2 ± 0.4	5.7 ± 0.5	11.0 ± 1.0
3	200 ppm AX/1.5% DSS	15	38.2 ± 3.3	2.2 ± 0.3	5.7 ± 0.5	11.4 ± 0.7
4	None	15	39.2 ± 3.1	2.2 ± 0.3	5.5 ± 0.3	13.2 ± 0.6

[a] Mean ± SD.

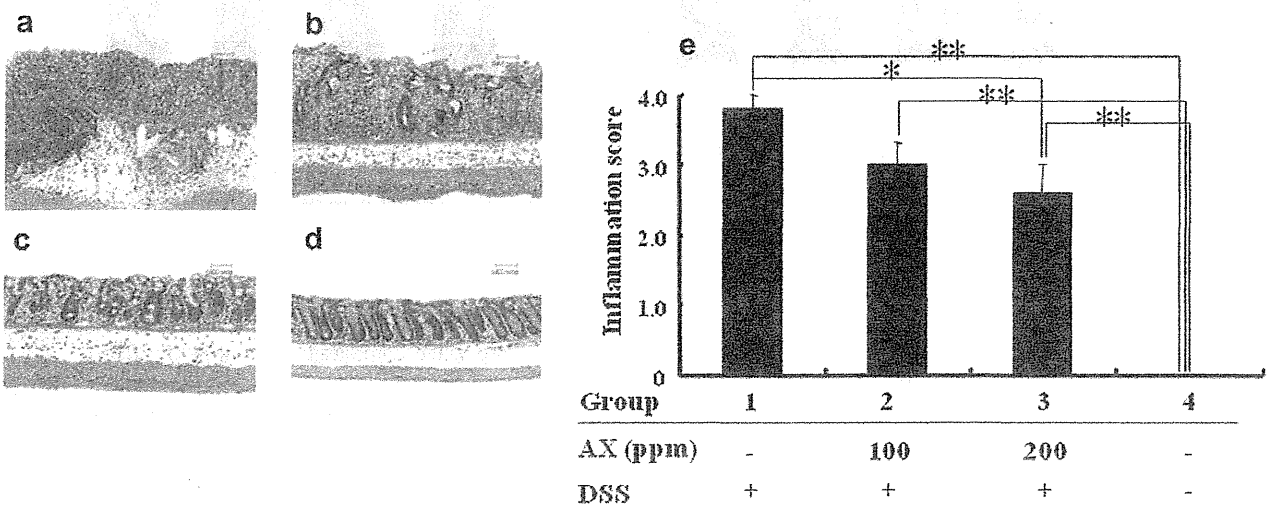

Fig. 4. Histopathological changes in the large bowel of a mouse from (a) DSS alone treated group, (b) DSS + 100 ppm AX group, (c) DSS + 200 ppm AX group, and (d) control. (f) Inflammatory scores in the large bowel of mice in all groups ($n = 5$). *, **: Significantly different at $P < 0.05$ and $P < 0.001$, respectively, by Tukey multiple comparison post test.

3.2.4. Immunohistochemical analysis of NF-κB in the colonic malignancies

Fig. 6 showed data on immunohistochemical score of NF-κB expression. The score of group 1 (1.5% DSS alone) was the greatest among the groups. AX feeding dose-dependently decreased the score and significant decreases were observed in the mice fed 100 ppm AX (group 2, $P < 0.001$) and 200 ppm AX (group 3, $P < 0.001$).

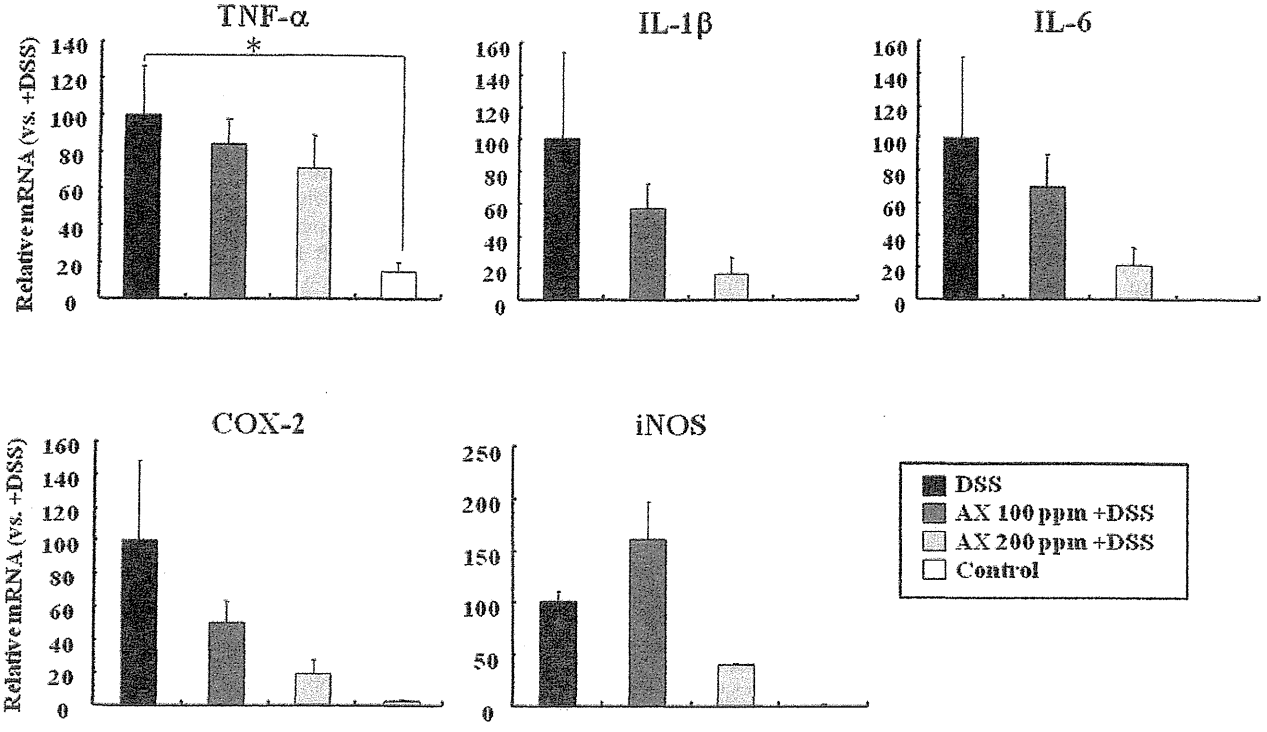

Fig. 5. The expression level of TNF-α, IL-1β, IL-6, COX-2, and iNOS mRNA in the large bowel of a mouse from all groups by RT-PCR analysis ($n = 5$). *: Significantly different at $P < 0.05$.

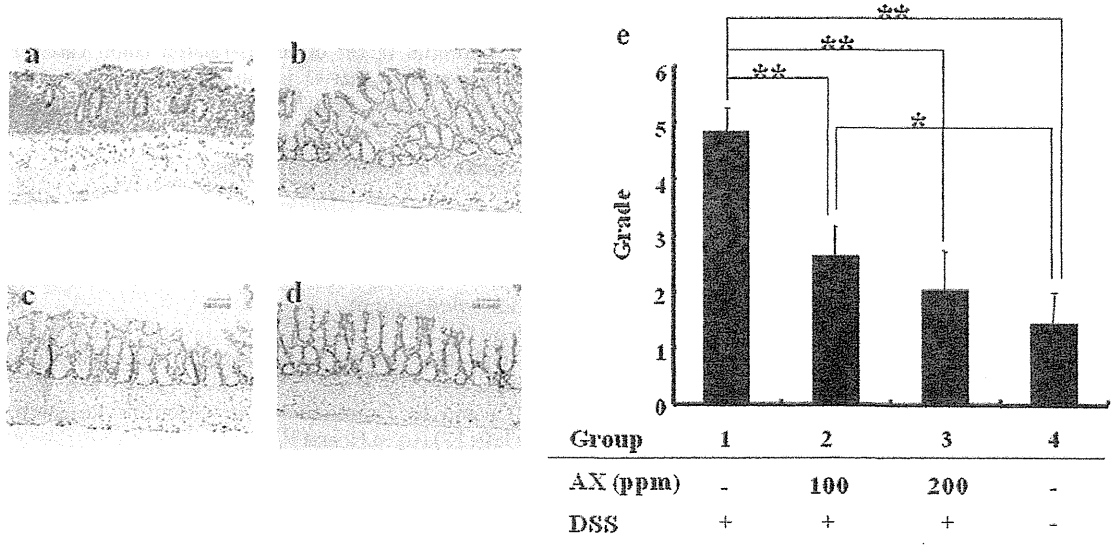

Fig. 6. Immunohistochemical analysis of NF-κB in the colon of a mouse from groups 1–4. (a) DSS alone treated group. (b) DSS + 100 ppm AX group. (c) DSS + 200 ppm AX group. (d) Control. (e) Quantitative analysis of NF-κB expression ($n = 5$). *,**: Significantly different at $P < 0.05$ and $P < 0.001$, respectively, by Tukey multiple comparison post test.

4. Discussion

Inflammation is an important tumor promoter [9,15], and several cytokines, such as TNF-α and IL-1β, induced by inflammation can promote tumor growth. Pro-inflammatory cytokines and chemokines are encoded by target genes of the NF-κB-activation pathway that are associated with tumor development and progression in human and mice [27]. Furthermore, many oncogenes and carcinogens cause activation of NF-κB, whereas chemicals with known chemopreventive properties can suppress NF-κB activation [28].

The effects of AX on tumor growth, cardiac function and immune response in mice have previously been reported [29,30]. AX enhances the cell-mediated immune responses. AX can prevent inflammatory processes by blocking the expression of pro-inflammatory genes as a consequence of suppressing the NF-κB activation in RAW264.7 cells and LPS-induced colitis model mice [29]. AX also suppresses the production of nitric oxide and prostaglandin E_2 and the pro-inflammatory cytokines TNF-α and IL-1β [29]. Moreover, AX could suppress endotoxin-induced uveitis by inhibiting NF-κB signaling in rats [30]. The authors also suggested that

the anti-inflammatory effects of AX through its suppression of NF-κB activation may be based on its antioxidant activity.

Numerous reports suggest that carotenoids, including AX, possess potent cancer chemopreventive properties [31–33]. Unfortunately, some major clinical trials of high-dose supplemental β-carotene, the carotenoid most frequently identified as protective against lung cancer, failed to demonstrate protection [34–37]. These findings suggest that the use of carotenoids without potential for conversion to vitamin A may provide protection and avoid this toxicity. In contrast, AX attracted considerable interest because of its potent anti-tumor, antioxidant and immunomodulatory activities, which are distinctly different and, at least in some cases, more potent than that of β-carotene and other carotenoids [38–41].

In this study, we demonstrated that dietary administration of three doses of AX ameliorated the AOM/DSS-induced colonic proliferative lesions (Tables 2 and 3) in mice. Suppression was prominent in mice treated with 200 ppm AX. The effects might be caused by inhibition of NF-κB expression as well as the expression of TNF-α and IL-1β in the malignancies (Fig. 3). Furthermore, AX suppressed the cell proliferation and the expression of survivin, which is a member of the inhibitor of apoptosis family (Fig. 2). In our previous study, 500 ppm AX and canthaxanthin suppressed the AOM-induced colon carcinogenesis in rats, and decreased cell proliferation activity [6]. In the current study, the lower doses (50, 100, and 200 ppm) of AX also suppressed mice colon carcinogenesis by modulating proliferation. We also observed the inhibitory effects of AX and canthaxanthin on rat oral carcinogenesis by suppressing proliferating activity [2]. Recently, Raju et al. [42] reported that high doses (1000 and 2000 ppm) of β-carotene and lutein augmented AOM-induced rat colonic aberrant crypt foci (ACF) formation, but low doses (100 or 200 ppm) of the chemicals significantly inhibited the ACF formation. Since AX as well as lutein is a xanthophyll carotenoid, the dose of AX needs the attention to set the high concentration of administration for chemoprevention trial.

NF-κB activation has been connected with multiple aspects of oncogenesis, including the control apoptosis, cell cycle, differentiation and cell migration [22,43]. Expression of several genes, such as COX-2, matrix metalloproteinase (MMP)-9, iNOS, TNF-α, IL-8, cell surface adhesion molecules and anti-apoptotic proteins which are involved in tumor initiation, tumor promotion and metastasis are regulated by NF-κB [23]. Recently, Nagendraprabhu et al. [8] reported that AX exhibited anti-cancer effects on DMH-induced rat colon carcinogenesis by inducing apoptosis and regulating the expressions of NF-κB, COX-2, MMPs-2/9, Akt, and ERK-2. A selective COX-2 inhibitor, etoricoxib, is reported to reduce the expression of NF-κB protein and inhibit DMH-induced colon ACF in rats [44]. Therefore, we suspected that AX may directly affect NF-κB and COX-2 expressions in the colonic mucosa and lesions.

We further studied the effect of AX on DSS-induced colitis in mice (Experiment 2). Dietary feeding with AX significantly suppressed several inflammatory events (Fig. 4) and NF-κB expression (Fig. 6) in colonic mucosa induced by DSS. Inflammatory genes, such as COX-2, iNOS, TNF-α, and IL-1β are the most common target genes participating in the activation of NF-κB signaling [45], and aberrant activation of NF-κB is associated with a number of chronic inflammatory diseases. In the current study, we observed decreases in the mRNA expressions of IL-1β, IL-6, COX-2, TNF-α, and iNOS in mice treated with AX and DSS when compared to that given DSS alone (Fig. 5), although the differences did not reach to statistical significance. Anti-inflammatory drugs may suppress the NF-κB pathway and suppression of NF-κB activation may be essential to prevent or treat several inflammatory diseases. Our findings thus suggest that AX suppressed the mouse colonic inflammation induced by DSS via modulating the NF-κB signaling pathway. The NF-κB signaling also has a major role in anti-apoptotic signaling and the development of cellular resistance against apoptosis [46]. Inhibition of apoptosis is mediated by the increased expression of inhibitor proteins of apoptosis (IAPs), such as c-FLIP, Bcl-xL, c-IAP1, c-IAP2 and XIAP. The resistance against apoptosis can expose dividing cells to tumorigenesis. Gupta et al. [47] reported that certain triterpenoids abrogated the expression of proteins associated with cell survival (Bcl-2, Bcl-xL, IAP-1, and IAP-2), proliferation (cyclin D1), invasion (MMP-9), and angiogenesis (VEGF), all regulated by NF-κB. It was suggested that that natural product inhibited the activation of NF-κB induced by carcinogens and inflammatory stimuli. Thus, NF-κB signaling represents the link between inflammation and cancer development and progression [45].

In the present study, although we discussed mainly the effect of AX on NF-κB pathway, it is also important to know that the interaction of AX with AOM and DSS-caused carcinogenesis may be involved in multiple signal pathways. Both AOM and DSS cause colon cancer through multiple signal pathways. AOM activated the Kras and thus its down-stream Mapk and Akt is activated. NF-κB and Bcl-2 are down-stream of Akt, TGF-β is decreased and β-catenin is increased [48]. DSS also related to alteration of multiple signal pathways [49]. Inhibition of pAkt reduced DSS-induced colitis [50]. Actually, AX has been shown to inhibit pAkt, bcl-2, bcl-xl in human colon cancer cells [4]. Thus, it is suggested that AX exerted the chemopreventive effect on colon carcinogenesis via multiple pathways, including NF-κB pathway.

Taken together, the results in the present study suggest that dietary AX inhibited the DSS-induced-inflammation and AOM/DSS-induced-colitis-associated colon carcinogenesis in mice through suppressing the expression of cytokines including NF-κB. Our findings indicate that NF-κB signal pathway may plays an important role in the colitis-associated colon carcinogenesis and is a potential target of colitis-related colon carcinogenesis. Our data also suggest that AX is one of the candidates of cancer chemopreventive agents against cancer development in inflamed colon.

5. Conflict of interest statement

The authors declare no financial or commercial conflict of interest.

Acknowledgement

This study was partly supported by Grants-in Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan (T.T.) and by Grants-in-Aid for Scientific Research, MEXT (No. 20380117) (to M.H. and Y.Y.).

References

[1] G. Hussein, H. Goto, S. Oda, U. Sankawa, K. Matsumoto, H. Watanabe, Antihypertensive potential and mechanism of action of astaxanthin: III. Antioxidant and histopathological effects in spontaneously hypertensive rats, Biol. Pharm. Bull. 29 (4) (2006) 684–688.
[2] T. Tanaka, H. Makita, M. Ohnishi, H. Mori, K. Satoh, A. Hara, Chemoprevention of rat oral carcinogenesis by naturally occurring xanthophylls, astaxanthin and canthaxanthin, Cancer Res. 55 (18) (1995) 4059–4064.
[3] M. Kurashige, E. Okimasu, M. Inoue, K. Utsumi, Inhibition of oxidative injury of biological membranes by astaxanthin, Physiol. Chem. Phys. Med. NMR 22 (1) (1990) 27–38.
[4] P. Palozza, C. Torelli, A. Boninsegna, R. Simone, A. Catalano, M.C. Mele, N. Picci, Growth-inhibitory effects of the astaxanthin-rich alga *Haematococcus pluvialis* in human colon cancer cells, Cancer Lett. 283 (1) (2009) 108–117.
[5] T. Tanaka, Y. Morishita, M. Suzui, T. Kojima, A. Okumura, H. Mori, Chemoprevention of mouse urinary bladder carcinogenesis by the naturally occurring carotenoid astaxanthin, Carcinogenesis 15 (1) (1994) 15–19.
[6] T. Tanaka, T. Kawamori, M. Ohnishi, H. Makita, H. Mori, K. Satoh, A. Hara, Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and

canthaxanthin during the postinitiation phase, Carcinogenesis 16 (12) (1995) 2957–2963.
[7] P.N. Prabhu, P. Ashokkumar, G. Sudhandiran, Antioxidative and antiproliferative effects of astaxanthin during the initiation stages of 1, 2-dimethyl hydrazine-induced experimental colon carcinogenesis, Fundam. Clin. Pharmacol. 23 (2) (2009) 225–234.
[8] P. Nagendraprabhu, G. Sudhandiran, Astaxanthin inhibits tumor invasion by decreasing extracellular matrix production and induces apoptosis in experimental rat colon carcinogenesis by modulating the expressions of ERK-2, NFκB and COX-2, Invest. New Drugs 29 (2) (2011) 207–224.
[9] T. Tanaka, Colorectal carcinogenesis: review of human and experimental animal studies, J. Carcinog. 8 (2009) 5.
[10] Y. Yasui, T. Tanaka, Chemoprevention of clolorectal carcinogenesis by natural anti-inflammatory agents, Anti-Inflammatory Anti-Allergy Agents Med. Chem. 9(b) (2010) 150–157.
[11] Y. Yasui, M. Kim, T. Oyama, T. Tanaka, Colorectal carcinogenesis and suppression of tumor development by inhibition of enzymes and molecular targets, Curr. Enzyme Inhib. 5 (2009) 1–26.
[12] N.P. Gullett, A.R. Ruhul Amin, S. Bayraktar, J.M. Pezzuto, D.M. Shin, F.R. Khuri, B.B. Aggarwal, Y.J. Surh, O. Kucuk, Cancer prevention with natural compounds, Semin. Oncol. 37 (3) (2010) 258–281.
[13] Y.J. Surh, Cancer chemoprevention with dietary phytochemicals, Nat. Rev. Cancer 3 (10) (2003) 768–780.
[14] S.C. Gupta, J.H. Kim, S. Prasad, B.B. Aggarwal, Regulation of survival, proliferation, invasion, angiogenesis, and metastasis of tumor cells through modulation of inflammatory pathways by nutraceuticals, Cancer Metastasis Rev. 29 (3) (2010) 405–434.
[15] T. Tanaka, H. Kohno, R. Suzuki, Y. Yamada, S. Sugie, H. Mori, A novel inflammation-related mouse colon carcinogenesis model induced by azoxymethane and dextran sodium sulfate, Cancer Sci. 94 (11) (2003) 965–973.
[16] Y. Yasui, R. Suzuki, S. Miyamoto, T. Tsukamoto, S. Sugie, H. Kohno, T. Tanaka, A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis, Int. J. Cancer 121 (10) (2007) 2331–2339.
[17] S. Miyamoto, Y. Yasui, T. Tanaka, H. Ohigashi, A. Murakami, Suppressive effects of nobiletin on hyperleptinemia and colitis-related colon carcinogenesis in male ICR mice, Carcinogenesis 29 (5) (2008) 1057–1063.
[18] T. Tanaka, Y. Yasui, M. Tanaka, T. Oyama, K.M. Rahman, Melatonin suppresses AOM/DSS-induced large bowel oncogenesis in rats, Chem. Biol. Interact. 177 (2) (2009) 128–136.
[19] H. Kohno, R. Suzuki, M. Curini, F. Epifano, F. Maltese, S.P. Gonzales, T. Tanaka, Dietary administration with prenyloxycoumarins, auraptene and collinin, inhibits colitis-related colon carcinogenesis in mice, Int. J. Cancer 118 (12) (2006) 2936–2942.
[20] H. Kohno, R. Suzuki, S. Sugie, T. Tanaka, Suppression of colitis-related mouse colon carcinogenesis by a COX-2 inhibitor and PPAR ligands, BMC Cancer 5 (2005) 46.
[21] G. Bonizzi, M. Karin, The two NF-kappaB activation pathways and their role in innate and adaptive immunity, Trends Immunol. 25 (6) (2004) 280–288.
[22] A.S. Baldwin, Control of oncogenesis and cancer therapy resistance by the
[31] H. Gerster, Beta-carotene, vitamin E and vitamin C in different stages of experimental carcinogenesis, Eur. J. Clin. Nutr. 49 (3) (1995) 155–168.
[32] P. Palozza, S. Serini, N. Maggiano, G. Tringali, P. Navarra, F.O. Ranelletti, G. Calviello, Beta-Carotene downregulates the steady-state and heregulin-alpha-induced COX-2 pathways in colon cancer cells, J. Nutr. 135 (1) (2005) 129–136.
[33] P. Palozza, S. Serini, F. Di Nicuolo, G. Calviello, Modulation of apoptotic signalling by carotenoids in cancer cells, Arch. Biochem. Biophys. 430 (1) (2004) 104–109.
[34] G.S. Omenn, G. Goodman, M. Thornquist, J. Grizzle, L. Rosenstock, S. Barnhart, J. Balmes, M.G. Cherniack, M.R. Cullen, A. Glass, et al., The beta-carotene and retinol efficacy trial (CARET) for chemoprevention of lung cancer in high risk populations: smokers and asbestos-exposed workers, Cancer Res. 54 (7 Suppl) (1994) 2038s–2043s.
[35] D. Albanes, O.P. Heinonen, P.R. Taylor, J. Virtamo, B.K. Edwards, M. Rautalahti, A.M. Hartman, J. Palmgren, L.S. Freedman, J. Haapakoski, M.J. Barrett, P. Pietinen, N. Malila, E. Tala, K. Liippo, E.R. Salomaa, J.A. Tangrea, L. Teppo, F.B. Askin, E. Taskinen, Y. Erozan, P. Greenwald, J.K. Huttunen, Alpha-Tocopherol, beta-carotene supplements, lung cancer incidence in the alpha-tocopherol, beta-carotene cancer prevention study: effects of base-line characteristics and study compliance, J. Natl. Cancer Inst. 88 (21) (1996) 1560–1570.
[36] G.S. Omenn, G.E. Goodman, M.D. Thornquist, J. Balmes, M.R. Cullen, A. Glass, J.P. Keogh, F.L. Meyskens Jr., B. Valanis, J.H. Williams Jr., S. Barnhart, M.G. Cherniack, C.A. Brodkin, S. Hammar, Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial, J. Natl. Cancer Inst. 88 (21) (1996) 1550–1559.
[37] C.H. Hennekens, J.E. Buring, J.E. Manson, M. Stampfer, B. Rosner, N.R. Cook, C. Belanger, F. LaMotte, J.M. Gaziano, P.M. Ridker, W. Willett, R. Peto, Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease, N. Engl. J. Med. 334 (18) (1996) 1145–1149.
[38] Y. Nakajima, Y. Inokuchi, M. Shimazawa, K. Otsubo, T. Ishibashi, H. Hara, Astaxanthin, a dietary carotenoid, protects retinal cells against oxidative stress in-vitro and in mice in-vivo, J. Pharm. Pharmacol. 60 (10) (2008) 1365–1374.
[39] P. Palozza, N.I. Krinsky, Astaxanthin and canthaxanthin are potent antioxidants in a membrane model, Arch. Biochem. Biophys. 297 (2) (1992) 291–295.
[40] Y.M. Naguib, Antioxidant activities of astaxanthin and related carotenoids, J. Agric. Food Chem. 48 (4) (2000) 1150–1154.
[41] E. Camera, A. Mastrofrancesco, C. Fabbri, F. Daubrawa, M. Picardo, H. Sies, W. Stahl, Astaxanthin, canthaxanthin and beta-carotene differently affect UVA-induced oxidative damage and expression of oxidative stress-responsive enzymes, Exp. Dermatol. 18 (3) (2009) 222–231.
[42] J. Raju, M.V. Swamy, I. Cooma, J.M. Patlolla, B. Pittman, B.S. Reddy, V.E. Steele, C.V. Rao, Low doses of beta-carotene and lutein inhibit AOM-induced rat colonic ACF formation but high doses augment ACF incidence, Int. J. Cancer 113 (5) (2005) 798–802.
[43] S.C. Gupta, C. Sundaram, S. Reuter, B.B. Aggarwal, Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy, Biochim. Biophys. Acta 1799 (10-12) (2010) 775–787.
[44] L. Tanwar, V. Vaish, S.N. Sanval, Chemoprevention of 1,2-dimethylhydrazine-

Cell Stress and Chaperones (2011) 16:653–662
DOI 10.1007/s12192-011-0280-8

ORIGINAL PAPER

High-dose green tea polyphenols induce nephrotoxicity in dextran sulfate sodium-induced colitis mice by down-regulation of antioxidant enzymes and heat-shock protein expressions

Hirofumi Inoue · Satoko Akiyama · Mari Maeda-Yamamoto · Atsushi Nesumi · Takuji Tanaka · Akira Murakami

Received: 2 June 2011 / Revised: 27 June 2011 / Accepted: 30 June 2011 / Published online: 16 July 2011

Abstract Previously, we reported that oral feeding of 1% green tea polyphenols (GTPs) aggravated the dextran sulfate sodium (DSS)-induced colitis in mice. In the present study, we assessed the toxicity of 1% GTPs in several organs from normal and DSS-exposed mice. Sixty-two male ICR mice were initially divided into four groups. Non-treated group (group 1, $n=15$) was given standard diet and water, GTPs (group 2, $n=15$) received 1% GTPs in diet and water, DSS (group 3, $n=15$) received diet and 5% DSS in water, and GTPs + DSS group (group 4, $n=17$) received 1% GTPs in diet and 5% DSS in water. We found that group 4 significantly increased ($P<0.05$) kidney weight, the levels of serum creatinine and thiobarbituric acid-reactive substances in both kidney and liver, as compared with those in group 3. The mRNA expression levels of antioxidant enzymes and heat-shock proteins (HSPs) in group 4 were lower than those of group 3. For instance, heme oxygenase-1 (HO-1), HSP27, and 90 mRNA in the kidney of group 4 were dramatically down-regulated as compared with those of group 3. Furthermore, 1% GTPs diet decreased the expression of HO-1, NAD(P)H:quinone oxidoreductase 1 (NQO1) and HSP90 in kidney and liver of non-treated mice. Taken together, our results indicate that high-dose GTPs diet disrupts kidney functions through the reduction of antioxidant enzymes and heat-shock protein expressions in not only colitis but also non-treated ICR mice.

Keywords Green tea polyphenols · Nephrotoxicity · Antioxidant enzyme · Heat shock protein · Colitis

H. Inoue · S. Akiyama · A. Murakami (✉)
Division of Food Science and Biotechnology,
Graduate School of Agriculture, Kyoto University,
Kyoto 606-8502, Japan
e-mail: cancer@kais.kyoto-u.ac.jp

M. Maeda-Yamamoto · A. Nesumi
National Institute of Vegetable and Tea Sciences,
National Agriculture and Food Research Organization,
2769 Kanaya,
Shimada, Shizuoka 428-8501, Japan

T. Tanaka
The Tohkai Cytopathology Institute: Cancer Research and Prevention (TCI-CaPP),
4-33 Minami-Uzura,
Gifu 500-8285, Japan

Inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, is a group of chronic disorders of the intestinal tract characterized by excessive production of reactive oxygen species (ROS) and cytokines (Araki et al. 2003). The etiology of IBD is believed to involve inappropriate host responses to complex commensal microbial flora in the gut and originates from mucosal barrier dysfunction, such as an abnormal leaky mucus layer, altered tight junction protein expression, and increased epithelial apoptosis (Araki et al. 2010).

To study the mechanisms of action underlying IBD, dextran sulfate sodium (DSS)-induced colitis models have been used in many laboratories including ours (Kwon et al. 2005). DSS exhibits toxic effects toward colonic epithelium and destroys the mucosal barrier, allowing bacteria to contact lamina propria cells (Kitajima et al. 1999a, b). Excess generation of ROS caused by the gut microenvironment breaks intestinal antioxidant systems in mice with DSS-induced colitis, thereby contributing to intestinal oxidative injury and initiating pro-inflammatory signaling

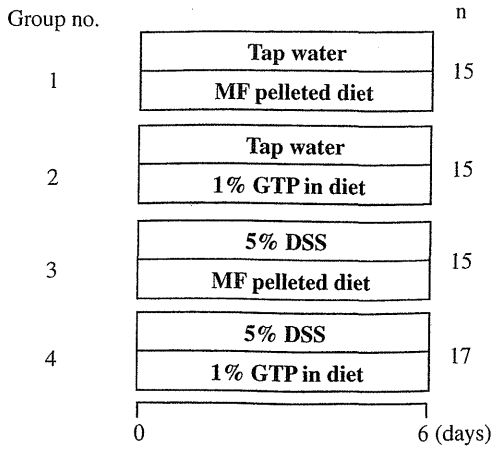

Fig. 1 Experimental groups. Experimental colitis was induced in male ICR mice by administrating 5% DSS in drinking water ad libitum throughout the experimental period. The non-treated group (group 1, n=15) was given tap water and a basal diet ad libitum, changed to fresh every day, for 6 days. The GTPs group (group 2, n=15) was given tap water and fed a diet containing 1% GTPs for 6 days. The DSS group (group 3, n=15) was fed a basal diet and given 5% DSS (*w/v*) in tap water for 6 days to induce colitis. The 1% GTPs+5% DSS group (group 4, n=17) was fed a diet containing 1% GTPs and given 5% DSS (*w/v*) in tap water for 6 days

MJ Research, Cambridge, MA) with mouse hypoxanthine phosphoribosyl transferase (HPRT), HO-1, NQO-1, MnSOD, GSTP1, HSP27, HSP70, and HSP90 primers, as follows: HPRT, 5′-GTAATGATCAGTCAACGGGGAC-3′ (forward) and 5′-CCAGCAAGCTTGCAACCTTAACCA-3′ (reverse); HO-1, 5′-TCCCAGACACCGCTCCTCCAG-3′ (forward) and 5′-GGATTTGGGGCTGCTGGTTTC-3′ (reverse); NQO1, 5′-TCGGAGAACTTTCAGTACCC-3′ (forward) and 5-GCAGAGAGTACATGGAGCC-3 (reverse); GSTP1, 5-TGCCACCGTACACCATTGTGT-3 (forward) and 5′-CAGCAGGTCCAGCAAGTTGTA-3′ (reverse); MnSOD, 5′-GCACATTAACGCGCAGTCA-3′ (forward) and 5′-AGCCTCCAGCAACTCTCCTT-3′ (reverse); HSP27, 5′-TGCTTCACCCGGAAATACAC-3′ (forward) and 5′-CTCGAAAGTAACCGGAATGG-3′ (reverse); HSP70, 5′-TGGTGCTGACGAAGATGAAG-3′ (forward) and 5′-AGGTCGAAGATGAGCACGTT-3′ (reverse); and HSP90, 5′-AAAGGCAGAGGCTGACAAGA-3′ (forward) and 5′-AGGGGAGGCATTTCTTCAGT-3′ (reverse). The PCR products were subjected to electrophoresis in 3% agarose gels and stained with 0.01% SYBR Gold (Molecular Probes, Eugene, OR). Band intensities were quantified using NIH image and no PCR saturation was confirmed. HPRT was used as the internal standard (Kwon et al. 2005).

AST, ALT, and creatinine measurements

Blood was collected from the inferior vena cava and serum was obtained by centrifugation at 3,000×*g* for 10 min at 4°C for analyses of biomarkers. AST, ALT, and creatinine were quantified using commercial kits (Wako Pure Chemical Industries).

Lipid peroxidation determined by measurement of TBARS

Kidney and liver samples (each ~25 mg) were homogenized in 250 μl of RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate) on ice. The homogenates were centrifuged at 1,600×*g* for 10 min at 4°C and the supernatants were subjected to assays. Lipid peroxidation in the kidneys and livers were assessed by measuring TBARS using a TBARS Assay kit (Cayman Chemical Company, Ann Arbor, MI).

Statistical analysis

The results are presented as the mean±standard deviation (SD) for each group. Statistical significance was assessed using one-way repeated ANOVA with a Tukey test. Differences were considered significant at $p<0.05$.

Results

General observations

We examined the effects of a 1% GTPs diet over a 6-day observation period. None of the mice in groups 1 and 2 died during the observation period, whereas 40% of the mice in group 3 (DSS only) and 60% in group 4 (GTPs + DSS) died by day 6 (Fig. 2a). Surprisingly, half of the mice in group 4 died by day 4. Mouse body weights in groups 3 and 4 began to decrease at 4 days after DSS exposure, and were significantly lower than those in groups 1 and 2 by day 6 (Fig. 2b). Time-dependent changes of both food and water intake showed tendencies similar to those of body weight (Fig. 2c, d).

Spleen, liver, and kidney weights

Colorectal length shortening reflects the extent of colonic damage in DSS-exposed mice (Okayasu et al. 1990). As shown in Fig. 3a, colorectal length in group 3 was shortened as compared to that in group 1, while GTPs supplementation did not have an effect (group 1 vs. 2, group 3 vs. 4). The spleen weight in group 3 was significantly greater (2.3-fold) as compared to group 1, whereas 1% GTPs in the diet suppressed that weight increase (Fig. 3b). Furthermore, liver weight in group 2 was significantly lower than that in group 1 (Fig. 3c). Notably, the kidney weight in group 4 was significantly increased (1.3-fold) as compared to group 3 (Fig. 3d).

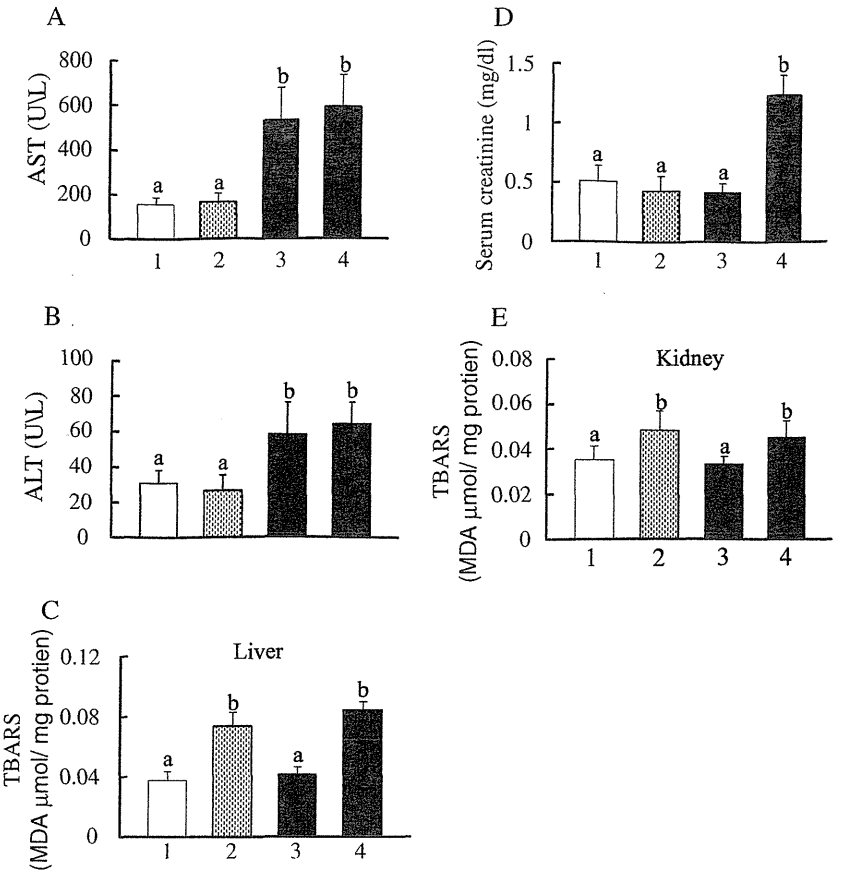

Fig. 4 Effects of DSS and/or 1% GTPs supplementation on hepatotoxicity and renal toxicity. Blood was collected and serum separated for determination of serum AST (**a**), ALT (**b**), and creatinine (**d**) levels. Obtained kidneys and livers were homogenized using RIPA buffer, then the supernatants were separated for measurement of TBARS levels in the livers (**c**) and kidneys (**e**). Data are shown as the mean±SD of seven to ten samples. *Bars with different letters* show significant differences ($P<0.05$)

Expression levels of antioxidant and xenobiotics metabolizing enzymes

We also determined whether DSS and/or GTPs supplementation had effects on the expression levels of antioxidant and xenobiotics metabolizing enzymes, including HO-1, NQO1, MnSOD, and GSTP1, in the kidneys and liver (Fig. 5a). Both renal and hepatic HO-1 mRNA expressions in group 2 were slightly but significantly decreased (19% and 9.6%, respectively) as compared with those in group 1. More strikingly, those expressions in group 4 (GTPs + DSS) were abolished (Fig. 5b). Furthermore, renal and hepatic NQO1 mRNA expressions in groups 2 and 3 were dramatically decreased as compared to those in group 1, while those in group 4 were abolished (Fig. 5c). Similar results were seen for GSTP1 (Fig. 5d). Although renal MnSOD expression in groups 2 and 3 was significantly lower than in group 1, there was no significant difference between groups 3 and 4 (Fig. 5e). Interestingly, the expression levels of hepatic MnSOD were consistent among the four groups (Fig. 5e).

Expression levels of HSPs

HSPs are induced in response to oxidative stress as well as heat shock (Tanaka et al. 2007; Bhattacharrya et al. 2009). Thus, we investigated whether 1% GTPs and/or DSS affect the expression levels of HSP70, HSP27, and HSP90 mRNA expressions in the mouse kidneys and livers (Fig. 6a). Although renal HSP70 expression in group 2 did not change as compared with that in group 1, it was abolished in groups 3 and 4 (Fig. 6b). On the other hand, 1% GTPs dramatically decreased hepatic HSP70 and HSP27 in groups 2, 3, and 4 (Fig. 6b, c). In contrast, as compared with group 1, renal HSP27 mRNA expression in groups 2 and 3 was not significantly different (Fig. 6c). It is interesting to note that renal HSP27 in group 4 was dramatically down-regulated as compared to the other three groups (Fig. 6c). Finally, as shown in Fig. 6d, renal and hepatic HSP90 mRNA expressions were the most sensitive to the GTPs diet (group 1 vs. 2, group 3 vs. 4).

Discussion

GTPs have a variety of beneficial health functions, including preventive effects toward diabetes and cancer. Moreover, numerous human intervention and bioavailability studies using green tea extracts or EGCG have reported no serious adverse effects from their use (Wang et al. 2008; Abboud et al. 2008; Mochizuki and Hasegawa 2010). On

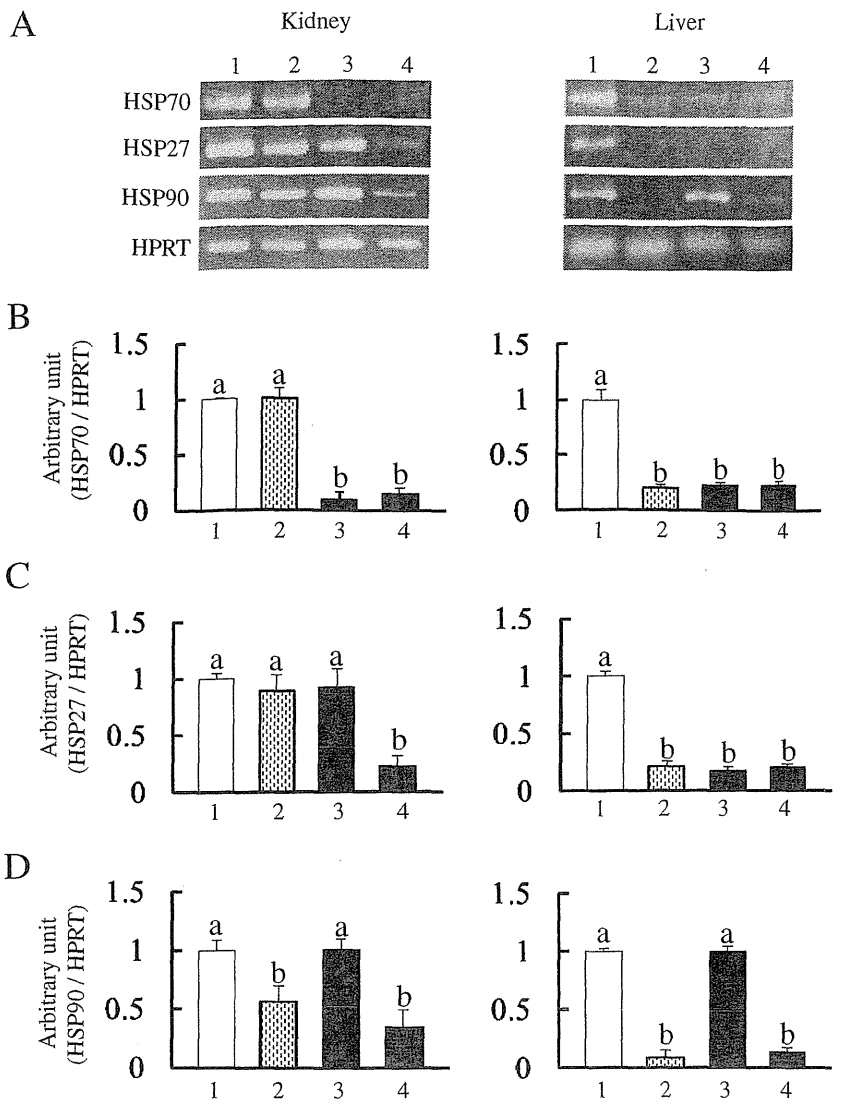

Fig. 6 Effects of DSS and/or 1% GTPs supplementation on heat-shock protein mRNA levels in mouse kidneys and livers. HSP70, HSP27, and HSP90 mRNA expressions were determined by RT-PCR (**a**), with representative findings presented. Densitometric quantification of HSP70 (**b**), HSP27 (**c**), and HSP90 (**d**) was performed using NIH Image. HPRT was used as an internal control. Data are shown as the mean±SD of ten samples. *Bars with different letters* show significant differences ($P<0.05$)

observed in the present study that a 1% GTPs diet did not aggravate liver function, as determined by serum AST and ALT levels (Fig. 4a, b). These contrasting results may be due to differences in experimental conditions. However, we also found that hepatic HSP mRNA expression levels were substantially down-regulated by the 1% GTPs diet in mice not treated with DSS (Fig. 6a, right panel), suggesting hepatic dysfunction based on essential roles of HSPs for homeostasis. Moreover, a diet containing both GTPs and DSS dramatically increased serum creatinine (Fig. 4d), the most reliable biomarker of nephropathy (Nakagawa et al. 2004; Yamabe et al. 2006). Notably, both green tea extracts and DSS have been found to be widely distributed throughout a variety of organs in mice, including the liver and kidneys (Suganuma et al. 1998; Kitajima et al. 1999a, b). Collectively, biological interplay between GTPs and DSS may have a crucial role in the development of hepato- and nephrotoxicity.

Oxidative stress is accelerated by a combination of ROS generation and impaired antioxidant capacity (Kankuri et al. 2003; Keshavarzian et al. 1992; Osburn et al. 2006). Previous pharmacological studies have shown that EGCG is metabolized through methylation, glucuronidation, and sulfation under normal physiological conditions, and then subsequently excreted in urine (Okushio et al. 1999; Li et al. 2001). It is important to note that, most, if not all, metabolites are biologically inactivated and are thus much less toxic than the intact form of EGCG. On the other hand, an excess dose of EGCG drastically increased the level of TBARS in mice, a reliable indicator of lipid peroxidation for exerting toxic effects and rapid lethality (Lambert et al. 2010). In line with these observations, the present 1% GTPs diet increased TBARS levels in the kidneys and liver (Fig. 4c, e), which may have a mechanistic association with its pro-oxidative property.