TABLE VI – EFFECT OF ONO-1714 ON THE SERUM BIOCHEMICAL PROFILES IN THE $Apc^{Min/+}$ AND $Apc^{+/+}$ MICE THAT RECEIVED DSS OR DSS + ONO-1714

Geno-type	Treatment (no. of mice)	Glu (mg/dl)	TP (g/dl)	Alb (g/dl)	A/G	T.Bil (mg/dl)	BUN (mg/dl)	T-Cho (mg/dl)	TG (mg/dl)
$Apc^{Min/+}$	1% DSS (12)	138.8 ± 41.9[1]	4.3 ± 0.3	1.4 ± 0.1	0.45 ± 0.03	0.3 ± 0.2	31.5 ± 6.5	92.0 ± 10.9	243.0 ± 115.5[2]
	1% DSS + 50 ppm ONO-1714 (10)	139.1 ± 72.1	4.2 ± 0.5	1.3 ± 0.2	0.44 ± 0.02	0.2 ± 0.2	30.8 ± 5.5	102.6 ± 16.8	112.5 ± 67.5[3]
	1% DSS + 100 ppm ONO-1714 (10)	127.6 ± 27.8	4.1 ± 0.3	1.2 ± 0.1	0.43 ± 0.02	0.1 ± 0.0	30.1 ± 6.5	106.0 ± 19.0	101.9 ± 75.7[3]
$Apc^{+/+}$	1% DSS (5)	166.9 ± 82.1	4.9 ± 0.5	1.6 ± 0.3	0.50 ± 0.10	0.3 ± 0.2	20.7 ± 5.4	80.4 ± 53.1	37.1 ± 32.2
	1% DSS DSS + 50 ppm ONO-1714 (5)	154.1 ± 66.7	4.7 ± 0.3	1.4 ± 0.1	0.44 ± 0.03	0.2 ± 0.2	22.8 ± 6.5	86.6 ± 48.4	37.4 ± 29.0
	1% DSS DSS + 100 ppm ONO-1714 (5)	196.0 ± 67.5	4.8 ± 0.4	1.4 ± 0.2	0.41 ± 0.03	0.1 ± 0.0	22.0 ± 3.4	96.0 ± 39.0	38.0 ± 21.0

[1]Mean ± SD.–[2]Significantly different from "1% DSS" group of $Apc^{+/+}$ mice by Bonferroni's multiple comparison post test ($^2p < 0.001$).–[3]Significantly different from the "1% DSS" group of $Apc^{Min/+}$ mice by Bonferroni's multiple comparison post test ($^3p < 0.01$).

suppress chemically-induced colitis,[9,23] AOM-induced colonic ACF development[30] and tumor load of $Apc^{Min/+}$ mice.[31] We also revealed that a highly selective inhibitor of iNOS, ONO-1714, suppresses AOM-induced ACF formation and decreased the volumes of colorectal tumor in rats.[18] In this study, the incidence of adenocarcinoma decreased by the treatment with ONO-1714, whereas that of adenoma was not affected. The reason for this is unknown, but it may be possible that feeding the rats ONO-1714 may have inhibited the progression of adenomas to adenocarcinomas. Since DSS strongly promoted the progression of dysplastic crypts, which are usually present in untreated $Apc^{Min/+}$ mice, to colonic malignancies,[8] ONO-1714 may thus blocks the progression. In our previous study, the expression of iNOS protein was remarkable in the invasion front of cancer tissue, and a COX-2 inhibitor suppressing this expression caused inhibition of tongue carcinogenesis.[32] Therefore, ONO-1714 may thus be able to potentially suppress cancer invasion.

It is well known that COX-2 expression is markedly elevated in the CRC of both human and rodents, and COX-2 plays an important role in cancer cell proliferation and tumor angiogenesis.[33] Moreover, COX-2 inhibition results in inhibition of CRC development, thus suggesting that the use of COX-2 inhibitors is protective against CRC occurrence.[34] Furthermore, we observed that a COX-2 selective inhibitor (nimesulide) exerts a powerful chemopreventive ability in AOM/DSS-induced colitis-related mouse colon carcinogenesis.[29] In addition, NO is reported to regulate activity and expression of COX-2.[35] iNOS inhibitors reduce not only iNOS activity but also COX-2 activity.[36] In the current study, COX-2 mRNA levels in the large intestine of DSS-treated $Apc^{Min/+}$ mice were much greater than those of wild-type mice. Furthermore, treatment with ONO-1714 reduced mRNA levels for COX-2 in the nonlesional mucosa of large intestine of $Apc^{Min/+}$ mice. Therefore, the down-regulation of COX-2 by an iNOS-inhibitor ONO-1714 might be one of the possible underlying mechanisms of suppression of large intestinal tumor development. Our findings also suggest that NO may regulate the production of prostaglandins, which are known to play a key role in colon tumor development, by affecting COX-2 expression.[37]

TNF-α and IL-1β are key cytokines involved in inflammation, immunity and cellular organization.[38] The colonic mucosa in UC patients produces large amounts of proinflammatory cytokines, such as TNF-α and IL-1β,[39] and increases productions of iNOS[11] and COX-2.[40] The increased production of these cytokines correlates with disease activity of IBD,[39] and their synthesis is implicated in the pathogenesis of the disease.[41] In experimental colitis, the expression of TNF-α and IL-1β is also enhanced.[42] Treatment with anti-TNFα monoclonal antibody revealed a more complex role for TNFα in colonic inflammation induced by DSS in mice.[43] Blockage of IL-1 activity decreases colitis in a rabbit model of colitis.[44] In this study, TNFα and IL-1β mRNA expression was substantially up-regulated in the colorectal mucosa of $Apc^{Min/+}$ mice that received DSS when compared with wild-type mice treated with DSS. The mechanisms by which the high-output NO generation through iNOS regulates cytokine release are not clear. However, in the current study, we found that ONO-1714 treatment reduces the mRNA expression of TNFα and IL-1β in the colonic mucosa of DSS-treated $Apc^{Min/+}$ mice. Since the induction of iNOS mRNA is gradual at the initial stage of colitis in $TNF\alpha^{-/-}$ mice when compared to $TNF\alpha^{+/+}$ mice,[45] interaction of iNOS, TNFα and IL-1β may play important roles in tumor promotion of inflammation-related carcinogenesis. Therefore, the suppression of the expression of TNFα and IL-1β by ONO-1714 contribute the low frequency of large tumors observed in this study.

Recent studies on experimental animal models of IBD have indicated that constitutive and inducible NO production seems to be beneficial during acute colitis, but the sustained up-regulation of NO is detrimental.[9] In this study, the results that the expression of iNOS mRNA in colorectal mucosa in both $Apc^{Min/+}$ and $Apc^{+/+}$ mice treated with DSS was increased by treatment with ONO-

1714 are of interest. The mechanisms for this are unclear, but the decreased production of NO by ONO-1714 may induce the expression of iNOS mRNA, because of the production of small quantities of NO maintain intestinal homeostasis and mucosal integrity.[9] Furthermore, NO is synthesized by not only iNOS but also by constitutive NOS, including endothelial NOS (eNOS) and neural NOS (nNOS).[9] The role of constitutively expressed NOS in intestinal inflammation is still not fully understood, but eNOS and nNOS isoforms may have influence on colitis, either by contributing to the inflammation or by affecting mucosal integrity in response to noxious stimuli.

Epidemiologically, a positive association between the hypertriglyceridemia and CRC development has been reported.[46] An experimental rodent study also showed a positive effect of serum TG on the development of ACF.[47] Niho *et al.*[48–50] recently discovered that a hyperlipidemic state is associated with intestinal polyp formation in *Apc*-deficient mice. They also observed that peroxisome proliferator-activated receptors' ligands[48,49] and lipoprotein lipase activator[50] reduce serum TG levels and suppress intestinal polyp formation in *Apc*-deficient mice. In accordance with their findings, the administration of ONO-1714 could therefore improve hyperlipidemia and suppress large bowel adenocarcinoma formation in the $Apc^{Min/+}$ mice in this study. Although we should investigate whether hyperlipidemia is a real risk factor in the CRC occurrence, our findings may suggest that an improvement of hyperlipidemia is beneficial in preventing colon carcinogenesis.

In conclusion, the dietary administration of ONO-1714 could effectively suppress colitis-related colon tumor development in the $Apc^{Min/+}$ mice by affecting multiple factors, including COX-2, TNFα, IL-1β and hyperlipidemia, which are involved in inflammation-related colon carcinogenesis. The clinical significance of our findings therefore merits further investigation.

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Diet supplemented with citrus unshiu segment membrane suppresses chemically induced colonic preneoplastic lesions and fatty liver in male *db/db* mice

Rikako Suzuki[1], Hiroyuki Kohno[1], Yumiko Yasui[1], Kazuya Hata[2], Shigeyuki Sugie[1], Shingo Miyamoto[1,3], Kuniaki Sugawara[4], Takashi Sumida[4], Yoshinobu Hirose[5] and Takuji Tanaka[1]*

[1]*Department of Oncologic Pathology, Kanazawa Medical University, Ishikawa, Japan*
[2]*BMR Laboratories, Sunplanet Co., Ltd., Gifu, Japan*
[3]*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan*
[4]*Research and Development, Ehime Beverage Inc., Matsuyama, Japan*
[5]*Department of Tumor Pathology, Graduate School of Medicine, Gifu University, Gifu, Japan*

The modulatory effects of dietary citrus unshiu segment membrane (CUSM) on the occurrence of aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCACs) were determined in male C57BL/KsJ-*db/db* (*db/db*) mice initiated with azoxymethane (AOM). Male *db/db*, *db/+* and +/+ mice were given 5 weekly subcutaneous injections of AOM (15 mg/kg body weight), and then they were fed the diet containing 0.02%, 0.1% or 0.5% CUSM for 7 weeks. At Week 12, a significant increase in the numbers of ACF and BCAC was noted in the *db/db* mice in comparison with the *db/+* and +/+ mice. Feeding with CUSM caused reduction in the frequency of ACF in all genotypes of mice and the potency was high in order of the *db/db* mice, *db/+* mice and +/+ mice. The number of BCACs was also reduced by feeding with CUSM, thus resulting in a 28–61% reduction in the *db/db* mice, possibly due to suppression of cell proliferation activity in the lesions by feeding with CUSM-containing diet. Clinical chemistry revealed a low serum level of triglyceride in mice fed CUSM. In addition, CUSM feeding inhibited fatty metamorphosis and fibrosis in the liver of *db/db* mice. Our findings show that CUSM in the diet has a chemopreventive ability against the early phase of AOM-induced colon carcinogenesis in the *db/db* as well as *db/+* and +/+ mice, indicating potential use of CUSM in cancer chemoprevention in obese people.

Key words: citrus unshiu segment membrane; ACF; BCAC; colon carcinogenesis; *db/db* mice

The modern Western lifestyle, including a high caloric intake, high-fat diets and physical inactivity, results in a positive energy balance, diabetes and obesity. These lifestyle patterns might also be risk factors for the development of colorectal cancer (CRC),[1] which is one of the major causes of morbidity and mortality in the Western world.[2] This malignancy has also increased in Asia owing to the changes in lifestyle, such as the dietary habit of increased meat consumption.[2,3] Several prospective and case-control studies have addressed the relationship between obesity/diabetes and CRC.[1,4,5]

C57BL/KsJ-*db/db* (*db/db*) mice are used as a genetically altered animal model with genotypes of obesity and diabetes mellitus.[6] A disruption of the leptin receptor (Ob-R) gene in these mice leads to an over-expression of leptin in the adipose tissue and a concomitantly high serum concentration of leptin.[7,8] The synthesis of leptin in adipocytes is influenced by insulin,[9] tumor necrosis factor-α,[10] glucocorticoids,[11] reproductive hormones[12] and prostaglandins[13] that may be involved in the neoplastic processes.[14] In addition, leptin can act as a growth factor in colonic epithelial cells[15] while modulating the proliferation of colonic cryptal cells.[16] In contrast, more leptin in the blood clearly decreased colon carcinogenesis in 3 different animal models.[17,18] Thus, leptin might be one of the biological factors involved in the development of CRC associated with obesity/diabetes. The *db/db* mouse, therefore, is a very useful model for elucidating the relationship between colon carcinogenesis and obesity/diabetes.

Certain food components are known to exert a cancer chemopreventive activity against CRC development.[19] However, few studies have so far been performed on the preventive effect of food components on obesity/diabetes-related colon carcinogenesis.[20,21] We recently have made the citrus unshiu segment membranes (CUSMs) that are rich in soluble and insoluble fiber and separate the juice vesiculates, from Satsuma mandarin (Citrus unshiu Marc.). Mandarin orange fruit constitutes 9–13 segments (juice sacs) that contain juice vesicles, and a membrane that wraps the segment is called "segment membrane." Although CUSM is waste product that remains after squeezing citrus unshiu for fruit juice, it contains biologically active compounds such as flavonoids, including hesperidin. Citrus fibers and flavonoids have been reported to inhibit colon carcinogenesis in rodents.[22–24] Obese individuals are thus often recommended to consume such diet low-energy foods rich in fiber with a possibly specific hypolipidemic effect, such as pectin-enriched dishes, fruit purees and juices and wheat bran bisquits.[25] Supplementation with flavonoids (hesperidin or naringin) improves the hyperglycemia in *db/db* mice.[26] In addition, CUSMs possess an antiobesity effect *in vitro* (Suzuki *et al.*, unpublished work). Although the biological activity of CUSM has yet to be elucidated, we suspected that CUSM might have a preventive effect on obesity/diabetes-related colon carcinogenesis.

In the current study, we determined the possible modulatory effects of dietary CUSM on the occurrence of azoxymethane (AOM)-induced aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCACs), which are putative precursor lesions for colonic adenocarcinoma,[27,28] in *db/db*, *db/+* and +/+ male mice. Since we previously observed the immunohistochemical overexpression of Ob-R and insulin-like growth factor-I receptor (IGF-1R) in AOM-induced BCACs in *db/db* mice,[29] the effects of CUSM on the expression of Ob-R and IGF-1R in BCACs and their surrounding cryptal cells were also investigated. Also, the effect of CUSM feeding on the cell proliferating activity of BCACs was assessed by counting proliferating cell nuclear antigen (PCNA)-index in the lesion.

Abbreviations: ACF, aberrant crypt foci; AOM, azoxymethane; BCACs, β-catenin accumulated crypts; CRC, colorectal cancer; CUSM, citrus unshiu segment membrane; IGF-1R, insulin-like growth factor-I receptor; H & E, hematoxylin and eosin; NF-κB, nuclear factor kappa B; Ob-R, leptin receptor; PCNA, proliferating cell nuclear antigen.

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***Correspondence to:** Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan. Fax: +81-76-286-6926. E-mail: takutt@kanazawa-med.ac.jp

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Material and methods

Animals, chemicals and diets

Four-week-old male *db/db* mice, *db/+* mice and +/+ mice were obtained from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained at the Kanazawa Medical University Animal Facility according to the Institutional Animal Care Guidelines, and were housed in polycarbonate cages (4–5 mice/cage) with free access to drinking water and a basal diet, MF (Oriental Yeast Co., Ltd., Tokyo, Japan), under controlled conditions of relative humidity [(50 ± 10)%], lighting (12-h light/dark cycle) and temperature [(23 ± 2) C]. AOM was purchased from the Sigma Chemical Co. (St. Louis, MO). Powdered CUSM was obtained from the Ehime Beverage Inc. (Matsuyama, Japan). The composition of CUSM (100 g) was as follows: 2.4 g moisture; 5.5 g protein; 0.3 g fat; 51 g fiber (22.0 g soluble and 29.0 g insoluble); 2.3 g ash; 26.6 g saccharide (6.1 g D-flucutose, 5.5 g glucose and 15.0 g D-suclose); 2.2 g hesperidin and 9.7 g others that include flavonoids, carotenoids and unknown components. The experimental diets were prepared by mixing CUSM into the basal diet at a dose of 0.02%, 0.1% or 0.5% on a weekly basis.

Experimental procedures

Male homozygous *db/db* mice (36 mice), heterozygous *db/+* mice (40 mice) and littermate controls (+/+) mice (40 mice) were divided into 4 groups, respectively. At 5 weeks of age, all mice were subcutaneously injected with AOM (15 mg/kg body weight) once a week for 5 weeks. Group 1 was fed the basal diet throughout the experiment. Groups 2 through 4 were fed the diets containing CUSM at dose levels of 0.02%, 0.1% and 0.5%, respectively, for 7 weeks, starting one week after the last injection of AOM. The experiment was terminated 12 weeks after the start.

All mice were provided with the experimental diets and tap water *ad libitum*, and were weighed weekly. The food intake of the animals was monitored every day. At the termination of the study (Week 12), all mice were sacrificed by an overdose of ether to analyze the number of AFC and BCACs. At autopsy, all organs, including the intestine, were carefully examined grossly, and then were examined histopathologically. The weighed liver and kidney were also submitted for histological examinations to investigate the toxicity of CUSM.

Identification of ACF and BCACs

The presence of ACF and BCACs was determined according to the standard procedures that are routinely used in our laboratory.[30,31] At necropsy, the colons were flushed with saline, excised, cut open longitudinally along the main axis and then washed with saline. They were cut, placed on the filter paper their mucosal surface up and then fixed in 10% buffered formalin for at least 24 hr. The fixed colons were stained with methylene blue (0.5% in distilled water) for 20 sec, dipped in distilled water and placed on a microscopic slide to count the ACF. After counting the ACF, the distal parts (1 cm from the anus) of the colon were cut in order to count the number of BCACs. To identify BCAC intramucosal lesions, the colon (0.58–0.87 cm^2/colon) was embedded in paraffin, and then a total of 20 serial sections (4-μm thick each) per mouse were made by an *en face* preparation.[32,33] For each case, 2 serial sections were used to analyze the BCACs.

Histopathology and immunohistochemistry

Five serial sections were made from paraffin-embedded blocks. Two sections were subjected to hematoxylin and eosin (H & E) staining for histopathology and β-catenin immunohistochemistry to count the number of colonic BCACs,[32,33] and others were used for Ob-R, IGF-1R and PCNA immunohistochemistry. Immunohistochemistry for β-catenin was performed on 4-μm-thick paraffin-embedded sections from the distal segments of the colons, using the labeled streptavidin-biotin method (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. The sections were treated for 40 min at room temperature with 2% bovine serum albumin and incubated overnight at 4 C with a primary antibody against β-catenin protein (diluted 1:1,000, Transduction Laboratories, Lexington, KY). Horseradish peroxidase activity was visualized by treatment with H_2O_2 and diaminobenzidine for 5 min. Negative control sections were immunostained without the primary antibody. Immunoreactivity was regarded as positive if apparent staining was detected in the cytoplasm and/or nuclei to determine the BCACs.

Immunohistochemistry of Ob-R and IGF-1R was performed using a stain system kit (Zymed, South San Francisco, CA). Rabbit polyclonal antibodies against Ob-R (1:200 dilution, sc-8325, Santa Cruz Biotechnology, Santa Cruz, CA) and IGF-1Rα (1:150 dilution, sc-7952, Santa Cruz Biotechnology) were applied overnight to the sections at 4 C according to the manufacturer's protocols. Human CRC samples were used as positive controls. The immunoreactivity cells were considered to be positive when definite cytoplasmic staining was identified. PCNA immunohistochemistry was performed on 4-μm-thick paraffin-embedded sections from colons of the *db/db* mice group by the labeled streptavidin biotin method using a LSAB KIT (DAKO Japan, Kyoto, Japan) with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65 C, deparaffinized in xylene and rehydrated through graded ethanol 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. Cells with intensively stained nuclei were considered to be positive for PCNA, and the indices (%) were calculated in each BCAC. Calculation was done in 20 BCACs from Group 9, 15 BCACs from Group 10, 10 BCACs from Group 11 and 8 BCACs from Group 12.

Morphometric analysis

Two serial sections from the liver of all mice were made for a morphometric analysis of liver fibrosis and fatty change. Liver sections were stained with H & E for histopathology and Sirius-red for morphometry of fibrosis. Fatty metamorphosis (% of fatty degeneration) was determined on the H & E-stained liver section, and liver fibrosis was expressed as the % of fibrosis in the area of liver section. An image analysis software, NIH Image v.1.63, was used for these calculates.

Clinical chemistry

At sacrifice, blood to measure the serum concentrations of glucose, leptin, insulin, cholesterol and triglyceride levels was collected from 5 mice, each of genotypes +/+, *db/+* and *db/db*. They were starved overnight prior to blood collection for clinical chemistry. The serum glucose level was measured enzymatically using the hexokinase method. The serum triglycerides were assayed by enzymatic hydrolysis with lipase. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. Serum concentrations of leptin and insulin were measured by an enzyme immunoassay according to the manufacturer's protocol (R & D systems, Minneapolis, MN).

Statistical evaluation

Where applicable, the data were analyzed using one-way ANOVA with Bonferroni correction or Fisher's exact probability test, with $p < 0.05$ as the criterion considered to indicate significance.

Results

General observations

The carefully monitored food intake of the animals showed that the mean daily intakes of *db/db* mice (7.26 g in the AOM alone group; 7.10 g in the AOM + 0.02% CUSM group; 7.22 g in the

TABLE I - BODY, LIVER, RELATIVE LIVER, EPIDIDYMAL FAT AND PANCREAS WEIGHTS IN EACH GROUP OF MALE MICE (+/+, *db*/+ AND *db*/*db*) THAT RECEIVED AOM AND CUSM

Group no.	Treatment	Body weight (g)	Liver weight (g)	Relative liver weight (g/100 g body wt.)	Epididymal fat weight (g)
1 (+/+)	AOM alone (9)	25.3 ± 1.1	1.23 ± 0.14	4.79 ± 0.42	0.39 ± 0.05
2 (+/+)	AOM + 0.02% CUSM (9)	23.8 ± 2.7	1.02 ± 0.20	4.25 ± 0.51	0.39 ± 0.15
3 (+/+)	AOM + 0.1% CUSM (9)	24.6 ± 1.3	1.15 ± 0.10	4.68 ± 0.29	0.37 ± 0.07
4 (+/+)	AOM + 0.5% CUSM (9)	24.7 ± 1.4	1.16 ± 0.06	4.70 ± 0.25	0.40 ± 0.11
5 (*db*/+)	AOM alone (10)	29.0 ± 1.5	1.29 ± 0.10	4.45 ± 0.26	0.65 ± 0.15
6 (*db*/+)	AOM + 0.02% CUSM (10)	29.6 ± 1.5	1.26 ± 0.18	4.24 ± 0.58	0.75 ± 0.12
7 (*db*/+)	AOM + 0.1% CUSM (10)	29.2 ± 1.2	1.25 ± 0.10	4.27 ± 0.28	0.74 ± 0.14
8 (*db*/+)	AOM + 0.5% CUSM (10)	28.9 ± 1.9	1.29 ± 0.11	4.47 ± 0.32	0.61 ± 0.14
9 (*db*/*db*)	AOM alone (10)	47.3 ± 5.0[1,2]	2.92 ± 0.53[3,4]	6.18 ± 0.73[3,4]	2.21 ± 0.45[1,2]
10 (*db*/*db*)	AOM + 0.02% CUSM (10)	46.6 ± 3.8[3,4]	2.79 ± 0.70[5,6]	5.96 ± 0.80[6,7]	2.28 ± 0.25[4,5]
11 (*db*/*db*)	AOM + 0.1% CUSM (10)	46.8 ± 7.3[5,6]	2.65 ± 0.47[1,2]	5.69 ± 0.92[1,2]	2.16 ± 0.50[6,7]
12 (*db*/*db*)	AOM + 0.5% CUSM (10)	45.2 ± 5.2[8,9]	2.47 ± 0.67[8,9]	5.43 ± 0.96[10]	2.28 ± 0.32[9,10]

All values are Mean ± SD.
Statistic analysis was done by Bonferroni Multiple Comparisons Test.
Values in parentheses in Column 2 indicate the number of mice examined.
[1]Significantly different from Group 4 ($p < 0.001$).–[2]Significantly different from Group 8 ($p < 0.001$).–[3]Significantly different from Group 2 ($p < 0.001$).–[4]Significantly different from Group 6 ($p < 0.001$).–[5]Significantly different from Group 3 ($p < 0.001$).–[6]Significantly different from Group 7 ($p < 0.001$).–[7]Significantly different from Group 3 ($p < 0.05$).–[8]Significantly different from Group 1 ($p < 0.001$).–[9]Significantly different from Group 5 ($p < 0.001$).–[10]Significantly different from Group 5 ($p < 0.05$).

AOM + 0.1% CUSM group and 7.25 g in the AOM + 0.5% CUSM group) were 1.25–1.34 times ($p < 0.01$ to $p < 0.001$) greater than other two genotypes (+/+ and *db*/+), regardless of treatments. The average body weights at the termination of the study were high in order of the *db*/*db* mice, the *db*/+ mice and the +/+ mice, as shown in Table I. Although the body weights of *db*/*db* mice were statistically higher ($p < 0.001$) than those of *db*/+ and +/+ mice, there was no significant difference among the treatment groups of each genotype. The liver and relative liver weights of *db*/*db* mice were greater than those of *db*/+ and +/+ mice, but the values did not significantly differ among the treatments groups of this genotype (Table I). The epididymal fat weight was heavy in the order of *db*/*db*, *db*/+ and +/+; the weight was insignificant among the treatment groups in each genotype (Table I). There were no significant differences regarding the mean pancreatic weight among the genotypes (data not shown). No clinical signs for the toxicity of CUSM were observed during the study.

Frequency of ACF and BCACs

At the end of the study, all the mice that received AOM developed colonic ACF and BCACs. Table II summarizes the data on colonic ACF formation. Regarding the mean number of ACF/colon in the AOM alone groups, the mean number of *db*/*db* mice was significantly higher ($p < 0.001$) than that of *db*/+ or +/+ mice. In comparison to the AOM alone group, the dietary administration with CUSM significantly reduced the number of ACF in all the genotypes: *db*/*db* mice, 53% reduction ($p < 0.001$) at a dose level of 0.02% CUSM, 54% reduction ($p < 0.001$) at a dose level of 0.1% CUSM and 59% reduction ($p < 0.01$) at a dose level of 0.5% CUSM; *db*/+ mice, 48% reduction ($p < 0.01$) at a dose level of 0.1% CUSM, 38% reduction ($p < 0.05$) at a dose level of 0.5% CUSM and +/+ mice, 45% reduction ($p < 0.05$) at a dose level of 0.1% CUSM and 62% reduction ($p < 0.001$) at a dose level of 0.5% CUSM. In addition, the percentages of ACF consisting of more than 4 aberrant crypts in all the CUSM-feeding groups in the *db*/*db* mice were significantly smaller (36% reduction by 0.02% CUSM, $p < 0.01$; 30% reduction by 0.1% CUSM, $p < 0.05$ and 47% reduction by 0.5% CUSM, $p < 0.001$) than that of AOM alone group (Table II). Although dietary administration with CUSM reduced the percentages of ACF consisting of more than 4 aberrant crypts in the *db*/+ and +/+ mice, the differences were insignificant.

BCACs also developed in the colon of all the genotypes of mice that received AOM alone, and the frequency per cm^2 of colonic mucosa was high in order of *db*/*db*, +/+ and *db*/+ mice (Table III). The dietary administration with CUSM at the highest dose (0.5%) significantly reduced the number of BCACs in the +/+ (65% reduction, $p < 0.05$) and *db*/*db* mice (74% reduction, $p < 0.001$). CUSM at a dose of 0.1% also significantly lowered the number of BCACs in *db*/*db* mice (53% reduction, $p < 0.001$).

Immunohistochemical analysis of Ob-R and IGF-1R

The immunohistochemical expression of Ob-R and IGF-1R was observed in the cytoplasm and nuclei of cryptal cells. Their expression was relatively strong in the nuclei of atypical cells in BCACs, when compared with their surrounding cryptal cells. Feeding with CUSM did not influence the stainability of Ob-R and IGF-1R (data not shown).

PCNA-labeling index

PCNA-labeling index was determined in BCACs that developed in the *db*/*db* mice (Groups 9 through 12). As illustrated in Figure 1, the mean PCNA-labeling indices of Group 11 (AOM + 0.1% CUSM, $p < 0.05$) and Group 12 (AOM + 0.5% CUSM, $p < 0.005$) were significantly lower than that of Group 9 (AOM alone). The values of Groups 9 and 10 (AOM + 0.02% CUSM) were comparable.

Histopathology and morphometric analysis in the liver

A histopathological examination of the liver revealed the occurrence of fatty metamorphosis [Fig. 2A-(*c*)] and fibrosis [Fig. 2B-(*c*)] in the *db*/*db* mice that received AOM alone, in contrast to the +/+ [Figs. 2A-(*a*) and 2B-(*a*)] and *db*/+ mice [Figs. 2A-(*b*) and 2B-(*b*)]. When the *db*/*db* mice were fed with 0.5% CUSM, these histopathological alterations (Fig. 3*a* and 3*b*) were inhibited ($p < 0.001$ for fatty metamorphosis and $p < 0.05$ for liver fibrosis).

Serum levels of cholesterol, triglycerides, glucose, insulin and leptin

The serum concentrations of total cholesterol, triglycerides, glucose, insulin and leptin are listed in Table IV. All the measurements in the *db*/*db* mice were higher than those of *db*/+ and +/+ mice. The dietary administration with CUSM did not significantly affect the serum levels of total cholesterol, glucose, insulin and leptin in all the genotypes. However, the serum level of triglycerides significantly decreased in the *db*/*db* mice ($p < 0.05$), when fed with the diet containing 0.5% CUSM (Table IV).

Discussion

The results of the current study confirmed the high susceptibility of AOM-induced colon carcinogenesis in the obese/diabetic *db*/*db* mice in our previous findings.[29] The high susceptibility in the *db*/*db* mice may be related to the increases in the body weight and the serum levels of total cholesterol, triglycerides, glucose, insulin and leptin, thus suggesting a positive association between

TABLE II - EFFECT OF CUSM ON AOM-INDUCED ACF FORMATION IN MALE MICE (+/+, *db*/+ AND *db*/*db*)

Group no.	Treatment	Total no. of ACF/colon	Total no. of ACs/colon	No. of ACs/focus	Total no. of 4 or more ACs/colon	% of ACF containing 4 or more ACs	No. of ACF/cm^2 of colon
1 (+/+)	AOM alone (9)	69 ± 12	228 ± 43	3.28 ± 0.35	23.20 ± 6.61	33.14 ± 6.57	10.78 ± 2.73
2 (+/+)	AOM + 0.02% CUSM (9)	48 ± 14 (30%)	153 ± 60 (33%)	3.14 ± 0.35 (4%)	15.60 ± 8.82 (33%)	30.83 ± 8.23 (7%)	8.16 ± 3.66 (24%)
3 (+/+)	AOM + 0.1% CUSM (9)	38 ± 5 (45%)[1]	108 ± 3 (53%)[2]	2.80 ± 0.33 (15%)	10.60 ± 4.04 (54%)	27.11 ± 8.02 (18%)	6.02 ± 0.79 (44%)
4 (+/+)	AOM + 0.5% CUSM (9)	26 ± 4 (62%)[3]	67 ± 11 (71%)[3]	2.61 ± 0.28 (20%)[1]	5.40 ± 1.82 (77%)[2]	20.95 ± 6.61 (37%)	4.02 ± 0.69 (63%)[2]
5 (*db*/+)	AOM alone (10)	77 ± 19	274 ± 50	3.60 ± 0.26	30.40 ± 7.44	40.00 ± 8.90[2]	11.99 ± 3.13
6 (*db*/+)	AOM + 0.02% CUSM (10)	53 ± 13 (31%)[4]	160 ± 34 (42%)[4]	3.02 ± 0.18 (16%)	16.20 ± 5.12 (47%)[5]	29.95 ± 3.94 (25%)	8.04 ± 2.39 (33%)[5]
7 (*db*/+)	AOM + 0.1% CUSM (10)	40 ± 6 (48%)[5]	121 ± 27 (56%)[5]	3.00 ± 0.32 (17%)	13.20 ± 3.03 (57%)[4]	32.63 ± 3.98 (18%)	6.53 ± 0.77 (46%)[5]
8 (*db*/+)	AOM + 0.5% CUSM (10)	48 ± 9 (38%)[5]	145 ± 32 (47%)[4]	2.98 ± 0.21 (17%)[5]	14.80 ± 4.76 (51%)[5]	30.51 ± 7.69 (24%)	7.62 ± 1.36 (36%)
9 (*db*/*db*)	AOM alone (10)	147 ± 23[3,6]	652 ± 88[3,6]	4.44 ± 0.34[3,6]	76.60 ± 10.90[3,6]	52.25 ± 6.02	20.83 ± 4.20[3,6]
10 (*db*/*db*)	AOM + 0.02% CUSM (10)	69 ± 10 (53%)[7]	206 ± 28 (68%)[7]	2.99 ± 0.07 (33%)[7]	23.00 ± 5.15 (70%)[7]	33.56 ± 6.39 (36%)[8]	8.37 ± 1.09 (60%)[7]
11 (*db*/*db*)	AOM + 0.1% CUSM (10)	68 ± 13 (54%)[7,9,10]	228 ± 45 (65%)[7,10,11]	3.33 ± 0.16 (25%)[7]	25.20 ± 6.91 (67%)[7,9]	36.35 ± 4.72 (30%)[12]	8.64 ± 2.90 (59%)[7]
12 (*db*/*db*)	AOM + 0.5% CUSM (10)	61 ± 6 (59%)[7,13]	179 ± 19 (73%)[7,14]	2.95 ± 0.19 (34%)[7]	16.80 ± 1.92(78%)[7]	27.85 ± 4.31 (47%)[7]	7.79 ± 1.30 (63%)[7]

All values are Mean ± SD.
Statistic analysis was done by Bonferroni Multiple Comparisons Test.
Values in parentheses in Column 2 indicate the number of mice examined.
Values in parentheses in Colounms 3–8 indicate inhibition rate.
[1]Significantly different from Group 1 ($p < 0.05$).–[2]Significantly different from Group 1 ($p < 0.01$).–[3]Significantly different from Group 1 ($p < 0.001$).–[4]Significantly different from Group 5 ($p < 0.01$).–[5]Significantly different from Group 5 ($p < 0.05$).–[6]Significantly different from Group 5 ($p < 0.001$).–[7]Significantly different from Group 9 ($p < 0.001$).–[8]Significantly different from Group 9 ($p < 0.01$).–[9]Significantly different from Group 3 ($p < 0.05$).–[10]Significantly different from Group 7 ($p < 0.05$).–[11]Significantly different from Group 3 ($p < 0.01$).–[12]Significantly different from Group 9 ($p < 0.05$).–[13]Significantly different from Group 4 ($p < 0.01$).–[14]Significantly different from Group 4 ($p < 0.05$).

TABLE III - EFFECT OF CUSM ON AOM-INDUCED BCAC FORMATION IN MALE MICE (+/+, *db*/+ AND *db*/*db*)

Group no.	Treatment	Total no. of BCAC/cm^2
1 (+/+)	AOM alone (9)	24.42 ± 7.26
2 (+/+)	AOM + 0.02% CUSM (9)	16.53 ± 4.88 (32%)
3 (+/+)	AOM + 0.1% CUSM (9)	10.52 ± 4.62 (57%)
4 (+/+)	AOM + 0.5% CUSM (9)	8.62 ± 3.91 (65%)[1]
5 (*db*/+)	AOM alone (10)	23.26 ± 8.53
6 (*db*/+)	AOM + 0.02% CUSM (10)	17.46 ± 4.87 (25%)
7 (*db*/+)	AOM + 0.1% CUSM (10)	14.84 ± 8.24 (36%)
8 (*db*/+)	AOM + 0.5% CUSM (10)	9.32 ± 3.86 (60%)
9 (*db*/*db*)	AOM alone (10)	41.94 ± 9.06[2,3]
10 (*db*/*db*)	AOM + 0.02% CUSM (10)	26.80 ± 10.52 (36%)
11 (*db*/*db*)	AOM + 0.1% CUSM (10)	19.84 ± 4.26 (53%)[4]
12 (*db*/*db*)	AOM + 0.5% CUSM (10)	11.10 ± 6.36 (74%)[4]

All values are Mean ± SD.
Statistic analysis was done by Bonferroni Multiple Comparisons Test.
Values in parentheses in Column 2 indicate the number of mice examined.
Values in parentheses in Column 3 indicate inhibition rate.
[1]Significantly different from Group 1 ($p < 0.05$).–[2]Significantly different from Group 1 ($p < 0.01$).–[3]Significantly different from Group 5 ($p < 0.01$).–[4]Significantly different from Group 9 ($p < 0.001$).

FIGURE 1 – PCNA labeling index in the BCACs that developed in the colon of *db*/*db* mice. Feeding with 0.1% ($p < 0.05$) and 0.5% CUSM ($p < 0.005$) significantly lowered the PCNA-labeling indices in the BCACs, but 0.02% CUSM feeding did not influence.

obesity/diabetes and colon tumorigenesis. Our findings also suggest that insulin resistance involves CRC development.[34] The main purpose of the current study was to investigate the effect of CUSM on the early phase of AOM-induced colon carcinogenesis in the *db*/*db* mice. Since the lesions ACF and BCACs are considered to be putative precursor lesions of colonic adenocarcinoma,[28,35] the results obtained clearly indicate the inhibitory effects of the dietary administration of CUSM on the development of AOM-induced ACF and BCACs in the *db*/*db* mice as well as the +/+ and *db*/+ mice. In the current study, all the serum measurements of total cholesterol, triglycerides, glucose, insulin and leptin were greater in the *db*/*db* mice than those of *db*/+ and +/+ mice, thus suggesting that these measurements may contribute to the high susceptibility of *db*/*db* mice to AOM-induced colon tumorigenesis. However, among the chemical profiles, only the triglyceride level lowered by feeding with CUSM correlated with a lower incidence of colonic preneoplastic lesions in the *db*/*db* mice. These

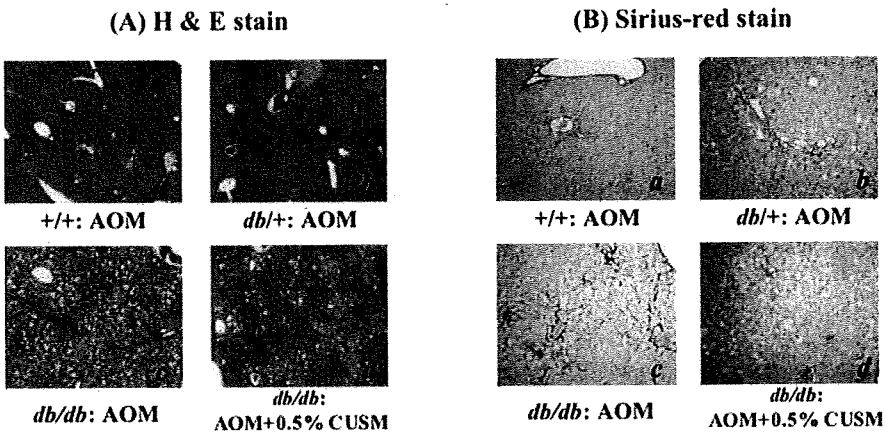

FIGURE 2 – Histopathology of liver. (A) There are numerous fat-containing vacuoles of varying size (the vacuoles are empty, the fat having dissolved in reagents) in the liver [A-(*c*)] of a *db/db* mouse receiving AOM, in contrast with those in the +/+ [A-(*a*)] and *db*/+ [A-(*b*)] mice treated with AOM. (B) Fibrosis (red) stained with Sirius-red is also evident in the liver [B-(*c*)] of a *db/db* mice that received AOM, in contrast with those in the +/+ [B-(*a*)] and *db*/+ [B-(*b*)] mice given AOM. These pathological alterations decreased after the administration of 0.5% CUSM in diet [A-(*d*) and B-(*d*)]. (A) H & E stain and (B) Sirius-red stain. Original magnification, (A) and (B) ×10. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

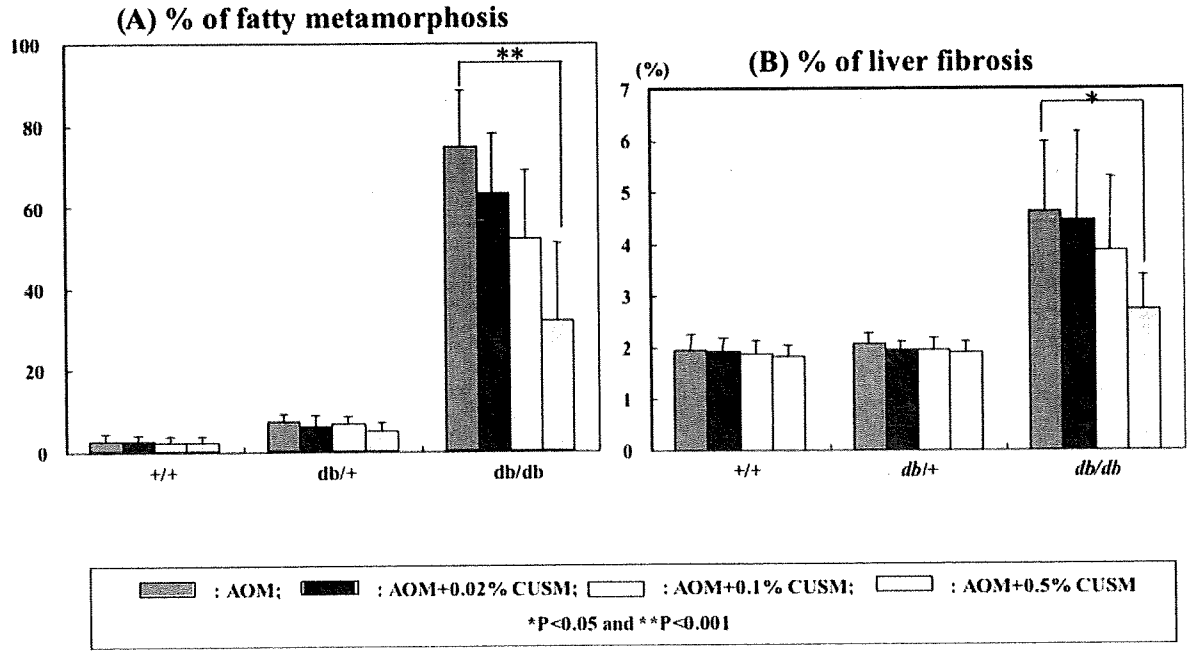

FIGURE 3 – A morphometric analysis of fatty metamorphosis and fibrosis in liver. Although the percentages of fatty metamorphosis (A) and fibrosis (B) in the liver of +/+ and *db*/+ mice were closely similar regardless of the administration of CUSM, the values were high in *db/db* mice. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

findings suggest that a high level of serum triglyceride is the most important biological effect for developing colonic tumors in *db/db* mice, and a modification (lowering) of this value may thus lead the inhibition of colon tumorigenesis. In fact, a positive association between the serum triglyceride levels and the risk of CRC development was found in humans.[36] This association was also suspected by the findings in animal experiments,[37] in which model animals for human familial adenomatous polyposis were used.[38]

Interestingly, feeding with a high-fat diet, which is implicated to play a role in the stimulation of colonic cryptal cell proliferation while also promoting colon carcinogenesis,[39] thus increases the circulating leptin level.[40] In addition, dietary fiber has been reported to decrease the serum leptin concentration while reducing colon carcinogenesis by lowering the degree of cryptal cell proliferation.[41] However, CUSM feeding did not affect serum leptin levels in the *db/db* mice. Thus, CUSM constituents other than fiber, i.e. flavonoids may contribute to the reduction in the occurrence of putative precursor lesions, ACF and BCACs in the colon of the *db/db* mice. We suspected that hesperidin in CUSM may therefore be responsible for the inhibition of preneoplasia development in male *db/db* mice, because this chemical can inhibit chemically-induced colon carcinogenesis in rodents.[19]

TABLE IV - SERUM PROFILES IN EACH GROUP OF MALE MICE (+/+, *db*/+ AND *db*/*db*) THAT RECEIVED AOM AND CUSM

Group no.	Treatment	Total cholesterol (mg/dL)	Triglycerides (mg/dL)	Glucose (mg/dL)	Insulin (ng/mL)	Leptin (ng/mL)
1 (+/+)	AOM alone (9)	90.2 ± 12.2	173.2 ± 31.6	224.6 ± 49.8	1.38 ± 0.87	11.4 ± 4.7
2 (+/+)	AOM + 0.02% CUSM (9)	93.2 ± 2.9	122.2 ± 33.8[1]	211.8 ± 19.6	1.13 ± 0.37	6.9 ± 1.5
3 (+/+)	AOM + 0.1% CUSM (9)	98.0 ± 11.5	137.6 ± 10.1	200.0 ± 30.2	1.59 ± 0.35	7.7 ± 1.6
4 (+/+)	AOM + 0.5% CUSM (9)	83.8 ± 11.0	116.4 ± 6.8[1]	228.2 ± 20.0	1.11 ± 0.12	5.3 ± 2.1
5 (*db*/+)	AOM alone (10)	108.8 ± 8.5	116.6 ± 7.0[1]	197.2 ± 15.0	2.01 ± 0.37	20.3 ± 10.1
6 (*db*/+)	AOM + 0.02% CUSM (10)	103.6 ± 4.4	134.4 ± 22.9	206.6 ± 17.6	4.49 ± 2.26	35.1 ± 7.2
7 (*db*/+)	AOM + 0.1% CUSM (10)	113.0 ± 8.6	107.2 ± 12.4[2]	211.6 ± 17.0	1.99 ± 0.38	21.7 ± 4.5
8 (*db*/+)	AOM + 0.5% CUSM (10)	104.8 ± 5.7	114.4 ± 10.7	206.2 ± 30.9	2.21 ± 0.77	14.2 ± 5.0
9 (*db*/*db*)	AOM alone (10)	149.0 ± 38.8[3]	192.4 ± 35.0[4]	685.0 ± 84.5[5,6]	13.89 ± 8.74[1,7]	244.2 ± 28.2[5,6]
10 (*db*/*db*)	AOM + 0.02% CUSM (10)	170.8 ± 39.0[8,9]	216.2 ± 39.3[10,11]	758.0 ± 71.3[8,9]	18.44 ± 9.56[8,9]	241.3 ± 42.8[9]
11 (*db*/*db*)	AOM + 0.1% CUSM (10)	180.3 ± 14.2[12,13]	157.8 ± 23.1[13]	641.5 ± 88.0[12,13]	18.66 ± 7.44[12,13]	260.0 ± 37.7[12,13]
12 (*db*/*db*)	AOM + 0.5% CUSM (10)	137.7 ± 33.1[14]	134.8 ± 15.3[15,16]	783.0 ± 84.1[17,18]	11.17 ± 7.47	252.8 ± 30.8[17,18]

All values are Mean ± SD.
Statistic analysis was done by Bonferroni Multiple Comparisons Test.
Values in parentheses in Column 2 indicate the number of mice examined.
[1]Significantly different from Group 1 ($p < 0.05$).–[2]Significantly different from Group 3 ($p < 0.05$).–[3]Significantly different from Group 1 ($p < 0.01$).–[4]Significantly different from Group 5 ($p < 0.01$).–[5]Significantly different from Group 1 ($p < 0.001$).–[6]Significantly different from Group 5 ($p < 0.001$).–[7]Significantly different from Group 5 ($p < 0.05$).–[8]Significantly different from Group 2 ($p < 0.001$).–[9]Significantly different from Group 6 ($p < 0.001$).–[10]Significantly different from Group 6 ($p < 0.01$).–[11]Significantly different from Group 2 ($p < 0.01$).–[12]Significantly different from Group 3 ($p < 0.001$).–[13]Significantly different from Group 7 ($p < 0.001$).–[14]Significantly different from Group 4 ($p < 0.01$).–[15]Significantly different from Group 9 ($p < 0.05$).–[16]Significantly different from Group 8 ($p < 0.05$).–[17]Significantly different from Group 4 ($p < 0.001$).–[18]Significantly different from Group 8 ($p < 0.001$).

An association between diabetes and cancer was suggested over 100 years ago.[42] The increased incidence of CRC in diabetic patients, mainly in those with type 2 diabetes, has been supported by a recent prospective, population-based cohort, case–control and meta-analysis studies.[5,43] Thus, there is an attractive hypothesis of insulin resistance-CRC, stating that insulin resistant may thus be associated with the development of CRC,[44] and this malignancy may therefore become a modifiable disease.[45] Regarding the mechanism of action, insulin resistance is associated with hyperinsulinemia, increased levels of growth factors, including IGF-1, and alterations in nuclear factor kappa B (NF-κB) and peroxisome proliferator-activated receptors signaling, which may promote CRC through their effect on the colonic cryptal cell kinetics.[21] Among these factors, insulin and the IGF axis may be related to CRC development.[46] IGF-1 may be able to influence both premalignant and cancer development. Similarly, insulin stimulates growth of normal colonic cryptal and cancer cells. Recently, an interesting finding indicated that leptin may interact with IGFs to promote survival and the expansion of colonic epithelial cells that were *Apc* deficient, but not those expressing wild-type *Apc*.[47] In the current study, feeding with CUSM did not influence the serum level of insulin and immunoreactivities of IGF-1R and Ob-R in the BCACs in the *db*/*db* mice. However, the treatment reduced cell proliferation activity in the BCACs by estimating PCNA-labeling index. CUSM could reduce the occurrence or progression of BCACs through lowering the cell proliferation, although the exact mechanism(s) should be elucidated.

In the present study, *db*/*db* mice treated with AOM had a greater incidence and multiplicity of ACF and BCACs. In addition, CUSM feeding inhibited fatty metamorphosis and fibrosis in the liver of *db*/*db* mice with hyperleptinemia treated with AOM. AOM is metabolically activated by CYP2E1.[48] Leptin treatment has been reported to increase the hepatic CYP2E1 expression in the *ob*/*ob* mutant mice.[49] The CYP2E1 activity that may increase due to AOM exposure and hyperleptinemia may therefore contribute to a higher incidence of putative precancerous lesions (ACF and BCACs) for CRC in the *db*/*db* mice. The inhibitory effects of CUSM on the development of putative precancerous lesions may be partly caused by influencing the hepatic and intestinal CYP2E1 activity,[50] but the content of fiber and/or pectin in the CUSM-containing diets was too low to exert their biological effects.

In summary, our data provide further evidence that *db*/*db* mice are susceptible to AOM-induced carcinogenesis[29] and such *db*/*db* mice can thus be an appropriate animal model for "metabolic syndrome," nonalcoholic fatty liver disease and/or nonalcoholic steatohepatitis.[51] Since our study focused on the effects of obesity and CUSM on the colonic premalignancies, not malignancies, further studies focusing the malignancies and NF-κB and IkappaB kinase[52,53] that can be activated through Ob-R1[14,44,46] and may thus play a critical role in obesity/diabetes-associated and colitis-related colon carcinogenesis in which processes the leptin involved[29] are needed for the prevention and treatment of the malignancies associated with these conditions.

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A lipophilic statin, pitavastatin, suppresses inflammation-associated mouse colon carcinogenesis

Yumiko Yasui[1], Rikako Suzuki[1], Shingo Miyamoto[2], Tetsuya Tsukamoto[3], Shigeyuki Sugie[1], Hiroyuki Kohno[1] and Takuji Tanaka[1]*

[1]*Department of Oncologic Pathology, Kanazawa Medical University, Uchinada, Ishikawa, Japan*
[2]*Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto, Japan*
[3]*Division of Oncological Pathology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya, Japan*

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors are known to modulate carcinogenesis. In this study, we investigated whether a lipophilic HMG-CoA reductase inhibitor pitavastatin suppresses inflammation-related mouse colon carcinogenesis. Male CD-1 (ICR) mice were initiated with a single intraperitoneal injection of azoxymethane (AOM, 10 mg/kg body weight) and promoted by 2% (w/v) dextran sodium sulfate (DSS) in drinking water for 7 days. The experimental diets containing pitavastatin at 2 dose levels (1 and 10 ppm) were fed to male CD-1 (ICR) mice for 17 weeks, staring 1 week after the cessation of DSS exposure. The effects of dietary pitavastatin on colonic tumor development were assessed at Weeks 5, 10 and 20. Feeding with pitavastatin at both doses significantly inhibited the multiplicity of colonic adenocarcinoma at Week 20. Furthermore, the treatment significantly lowered the positive rates of proliferating cell nuclear antigen and increased the apoptotic index in the colonic epithelial malignancies. The treatment also reduced nitrotyrosine-positivity in the colonic mucosa. Our findings thus show that pitavastatin is effective in inhibiting colitis-related colon carcinogenesis through modulation of mucosal inflammation, oxidative/nitrosative stress, and cell proliferation.

Key words: statin; chemoprevention; inflammation; colon carcinogenesis; mouse

Statins, which are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, are commonly-used drugs for the treatment of hypercholesterolemia.[1,2] They are able to decrease low-density lipoprotein (LDL) cholesterol levels by inhibiting HMG-CoA reductase. Furthermore, a triglyceride (TG)-lowering effect and a high-density lipoprotein (HDL) cholesterol-raising effect were observed in patients with hyperlipidemia, who take statins.[3,4] Statins have multibiological effects other than antilipidemia. Recently, it has been highlighted that statins are linked with several beneficial effects beyond their effect on cardiovascular disease. They include reduction in the risk of dementia,[5,6] fracture[7] and cancer.[8–10] Several recent preclinical studies indicated that statins may have chemopreventive potential against cancer at various sites,[10–12] including colon.[13–16] In addition, there is growing evidence that statins exert anti-inflammatory and antioxidative actions that are independent of their serum lipid lowering effects.[17]

Association between inflammation and cancer has long been suspected.[18,19] An example is that inflamed colon is a high risk for colorectal cancer (CRC) development.[20] CRC is thus one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC)[20] and Crohn's disease (CD).[21] For understanding the pathogenesis of IBD and IBD-related CRC, we have developed a novel colitis-related and two-stage mouse CRC model, using a colon carcinogen azoxymethane (AOM) and a colitis-inducing agent dextran sodium sulfate (DSS).[22] In this animal model, numerous large bowel adenocarcinomas occur within a short-term period, and their histology and biological alterations resemble those found in human.[22] The model can be used for investigating and determining cancer chemopreventive agents against CRC[23] as well as initiating or modulating agents for CRC.[24]

A lipophilic statin pitavastatin, (+)-monocalcium bis{(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate} ($C_{50}H_{46}CaF_2N_2O_8$, MW 880.98, Fig. 1), that has been developed in Japan is highly effective for lowering serum cholesterol and TG levels.[25] The lowering effect of pitavastatin on serum LDL-cholesterol is more potent than that of pravastatin, simbastatin and atorvastatin.[26–28] The drug possessing a high oral bioavailability is only slightly metabolized, suggesting a longer duration of action and is less potent for drug interactions.[28] Therefore, the agent is currently undergoing Phase III trials in Europe, US and Japan.[25] Since pitavastatin possesses pleiotrophic biological effects, including anti-inflammatory actions,[29,30] we in the present study investigated the potential chemopreventive ability of colitis-related colon cancer development using our mouse model[22] to find desirable cancer chemopreventers against IBD-related CRC.[31] Since numerous evidence demonstrates that a high-fat diet is associated with the risk of CRC development and serum levels of TG and cholesterol are positively associated with colon carcinogenesis,[32] we monitored serum levels of TG and cholesterol during the study.

Material and methods

Animals, chemicals and diets

Male Crj: CD-1 (ICR) mice (Charles River Japan, Tokyo, Japan) 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/cages) with free access to drinking water and pelleted basal diet, CRF-1 (Oriental Yeast, Tokyo, Japan), under controlled conditions of humidity (50 ± 10%), light (12/12 hr light/dark cycle) and temperature (23 ± 2°C). After arrived, animals were quarantined for the first 7 days, and then randomized by their body weights into experimental and control groups. A colonic carcinogen AOM was purchased from Sigma Chemical (St. Louis, MO). DSS with a molecular weight of 36000–50000 (Cat. No. 160110) was purchased from MP Biomed-

Abbreviations: AOM, azoxymethane; CD, Crohn's disease; CRC, colorectal cancer; DSS, dextran sodium sulfate; H&E, hematoxylin and eosin; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; IBD, inflammatory bowel disease; iNOS, inducible bitric oxide synthase; LDL, low-density lipoprotein; NF-κB, nuclear factor-kappa B; NO, nitric oxide; PCNA, proliferating cell nuclear antigen; PSC, primary sclerosing cholangitis; ssDNA, single-stranded DNA; TG, triglycerides; UC, ulcerative colitis; UDCA, ursodeoxycholic acid.

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***Correspondence to:** Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan. Fax: +81-76-286-6926. E-mail: takutt@kanazawa-med.ac.jp

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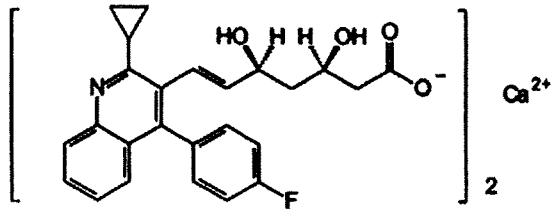

FIGURE 1 – Chemical structure of pitavastatin. (+)-Monocalcium bis{(3*R*,5*S*,6*E*)-7-[2-cyclopropyl-4-(4-fluorophenyl)-3-quinolyl]-3,5-dihydroxy-6-heptenoate}, $C_{50}H_{46}CaF_2N_2O_8$, MW 880.98.

icals, LLC (Aurora, OH). DSS for induction of colitis was dissolved in water at a concentration of 2% (w/v).

Experimental procedures

A total of 132 male ICR mice were divided into 7 experimental and control groups (Fig. 2). Mice in Groups 1–3 were given a single intraperitoneal injection of AOM (10 mg/kg body weight). Starting 1 week after the injection, animals received 2% DSS in the drinking water for 7 days. Subsequently, they were fed the diets containing 0, 1 and 10 ppm pitavastatin for 17 weeks, respectively, starting 1 week after the cessation of DSS exposure. Group 4 was fed the diet containing 10 ppm pitavastatin, and received no further treatments. Groups 5 and 6 were given AOM alone and DSS alone, respectively. Group 7 was an untreated control. Animals are sequentially sacrificed at Weeks 5, 10 and 20 by ether overdose to determine the effects of pitavastatin on colon tumorigenesis and biochemical profiles, including serum lipids measurements. Prior to sacrifice, animals were starved overnight for clinical chemistry. At sacrifice, the large bowels were flushed with saline, and excised. After measuring their length (from the ileocecal junction to the anal verge), large bowels were cut open longitudinally along the main axis, and gently washed with saline. The whole large bowel was macroscopically inspected for the presence of tumors, cut along a vertical axis and fixed in 10% buffered formalin for a least 24 hr. Histopathological examination was performed on paraffin-embedded sections after hematoxylin and eosin (H&E) staining. On H&E-stained sections, pathological lesions, such as mucosal ulceration, dysplasia and colonic tumors, were determined.

Clinical chemistry

At autopsy, whole blood anticoagulated with heparin lithium was taken from the inferior vena cava with a sterile syringe (Terumo, Tokyo, Japan) at each time point. The serum was obtained by centrifugation (3,000 rpm for 10 min), and stored at −80°C until measurement. Serum cholesterol was determined enzymatically using cholesterol esterase and cholesterol oxidase. The serum TG was assayed by enzymatic hydrolysis with lipase. These measurements were expressed as mg/dL.

Scoring of inflammation in the large bowel

Inflammation in the large bowel was scored on the H&E-stained sections. For scoring, large intestinal inflammation was graded according to the following morphological criteria described by Cooper *et al.*[33]: 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 the entire crypts with severe inflammation in the mucosa and submucosa, but with retainment of the surface epithelium; 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 made on the entire colon with or without proliferative lesions and expressed as a mean average score/mouse.

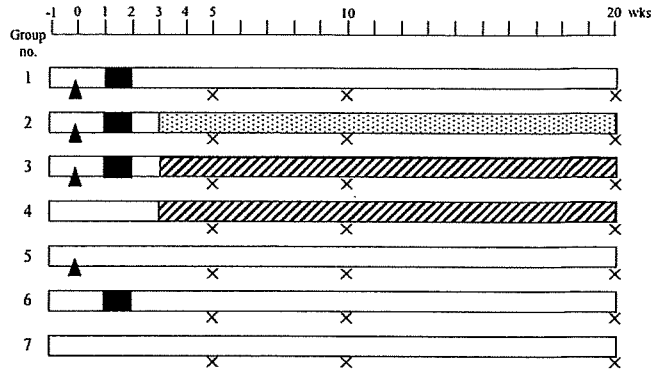

FIGURE 2 – Experimental protocol. ▲: AOM (10 mg/kg i.p.); ■: 2% DSS in drinking water; □: Basal diet and tap water; ⬚: 1 ppm pitavastatin in diet; ▨: 10 ppm pitavastatin in diet; ×: Sacrifice.

Immunohistochemistry

Immunohistochemistry for proliferating cell nuclear antigen (PCNA)-positive nuclei, apoptotic nuclei, and nitrotyrosine-positive cells was performed on 4-μm-thick paraffin-embedded sections, from the colons of mice in each group by the labeled strepravidin biotin method, using a LSAB KIT (DAKO Japan, Kyoto, Japan), with microwave accentuation. The paraffin-embedded sections were heated for 30 min at 65°C, deparaffinized in xylene and rehydrate through grade ethanols 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. For the determination of PCNA-incorporated nuclei, the PCNA-immunohistochemistry was performed. Apoptotic index was also evaluated by immunohistochemistry for single-stranded DNA (ssDNA). Sections were treated for 40 min at room temperature, with 2% BSA, and incubated overnight at 4°C with primary antibodies, anti-PCNA mouse monoclonal antibody (PC10, 1:50 dilution, DAKO Japan), anti-ssDNA rabbit polyclonal antibody (1:300 dilution, DAKO Japan) and anti-nitrotyrosine rabbit polyclonal antibody (1:500 dilution, Update Biotechnology, Lake Placid, NY). To reduce the nonspecific staining of mouse tissue by a mouse antibody (anti-PCNA), a Mouse On Mouse IgG blocking reagent (Vector Laboratories, Burlingame, CA) was applied for 1 hr. House-radish peroxidase activity was visualized by 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, negative controls were performed on serial sections. On the control sections, incubation with the primary antibodies was omitted.

Intensity and localization of immunoreactivity against all primary antibodies used were assessed using a microscope (Olympus BX41, Olympus Optical, Tokyo, Japan). The indices for PCNA and apoptosis were determined by counting the number of positive nuclei among at least 200 cells in 5 adenocarcinomas developed at Week 20 from each of Groups 1–3, and were indicated as percentages. The nitrotyrosine-positive cells were evaluated for their intensity of immunoreactivity on a 0 or 4+ scale. The overall intensity of the staining reaction was scored with 0 indicating no immunoreactivity and no positive cells, 1+ weak immunoreactivity and <10% of positive cells, 2+ mild immunoreactivity and 10–30% of positive cells, 3+ moderate immunoreactivity and 31–60% of positive cells and 4+ strong immunoreactivity and 61–100% of positive cells. This evaluation was done on the colonic mucosa with or without tumors from all the mice of each sacrifice time point (4 mice each from all groups at Week 5; 4 mice each from all groups at Week 10; and 9 mice each of Groups 1 and 3, 10 mice each of Groups 2 and 6, and 5 mice each of Groups 4, 5 and 7 at Week 20).

TABLE I – BODY, LIVER WEIGHT AND LENGTH OF LARGE BOWEL 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/2% DSS (9)	44.02 ± 3.44[a]	2.45 ± 0.34	5.56 ± 0.44	11.63 ± 0.41
2	AOM/2% DSS/1 ppm pitavastatin (7)	43.09 ± 6.79	2.21 ± 0.26	5.15 ± 0.28	11.66 ± 0.61
3	AOM/2% DSS/10 ppm pitavastatin (9)	38.40 ± 2.61	2.28 ± 0.32	5.94 ± 0.64[b]	11.29 ± 0.86
4	10 ppm pitavastatin (5)	42.47 ± 4.17	2.28 ± 0.23	5.39 ± 0.27	11.70 ± 1.54
5	AOM (5)	53.26 ± 6.63[c]	2.50 ± 0.40	4.68 ± 0.38[d]	12.18 ± 0.47
6	2% DSS (7)	44.16 ± 5.12	2.45 ± 0.30	5.59 ± 0.76	11.13 ± 0.28
7	None (4)	42.84 ± 4.23	2.40 ± 0.32	5.58 ± 0.23	12.78 ± 0.17

[a]Mean ± SD.–[b]Significantly different from Group 2 by Tukey–Kramer multiple comparison post test ($p < 0.05$).–[c]Significantly different from Groups 1, 6, and 7 by Tukey–Kramer multiple comparison post test ($p < 0.05$).–[d]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.05$).

TABLE II – INCIDENCE OF COLONIC LESIONS AT WEEKS 5, 10 AND 20

Group no.	Treatment (no. of mice examined at wk 5/wk 10/wk 20)	Mucosal ulcer			Dysplasia		
		Wk 5	Wk 10	Wk 20	Wk 5	Wk 10	Wk 20
1	AOM/2% DSS (4/4/9)	4/4, 100%	4/4, 100%	6/9, 67%	4/4, 100%	4/4, 100%	9/9, 100%
2	AOM/2% DSS/1 ppm pitavastatin (4/4/10)	4/4, 100%	3/4, 75%	3/10, 30%	4/4, 100%	4/4, 100%	8/10, 80%
3	AOM/2% DSS/10 ppm pitavastatin (4/4/9)	2/4, 50%	3/4, 75%	0/9, 0%	3/4, 75%	4/4, 100%	9/9, 100%
4	10 ppm pitavastatin (4/4/5)	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%
5	AOM (4/4/5)	0/4, 0%	1/4, 25%	0/5, 0%	1/4, 25%	0/4, 0%	0/5, 0%
6	2% DSS (4/4/10)	4/4, 100%	4/4, 100%	0/10, 0%	1/4, 25%	0/4, 0%	0/10, 0%
7	None (4/4/5)	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%

Data were from histopathological analysis.

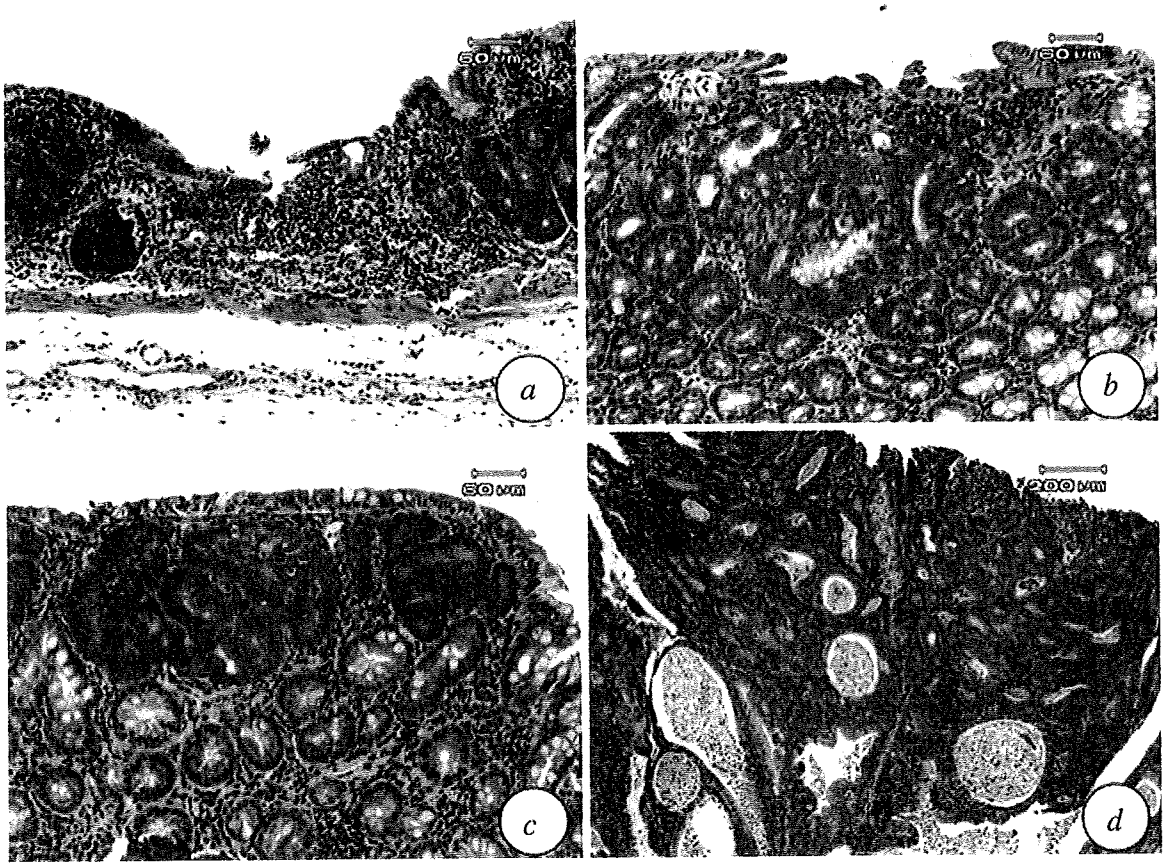

FIGURE 3 – Colonic lesions induced by AOM and 2% DSS. (*a*) A mucosal ulcer, (*b*) dysplastic crypts, (*c*) a tubular adenomas and (*d*) a tubular adenocarcinoma that developed in a mouse that received AOM and 2% DSS (Group 1). Bars inserted are (a) 60 μm, (b) 60 μm, (c) 60 μm and (d) 200 μm.

TABLE III – MULTIPLICITIES OF COLONIC LESIONS AT WEEKS 5, 10 AND 20

Group no.	Treatment (no. of mice examined at wk 5/wk 10/wk 20)	Mucosal ulcer			Dysplasia		
		Wk 5	Wk 10	Wk 20	Wk 5	Wk 10	Wk 20
1	AOM/2% DSS (4/4/9)	3.25 ± 1.71[a]	2.75 ± 0.96	0.82 ± 0.98	4.50 ± 1.30	4.00 ± 1.41	3.18 ± 1.66
2	AOM/2% DSS/1 ppm pitavastatin (4/4/10)	2.00 ± 0.82	1.25 ± 0.96	1.50 ± 2.46	3.25 ± 0.50	3.30 ± 1.30	2.20 ± 1.99
3	AOM/2% DSS/10 ppm pitavastatin (4/4/9)	1.25 ± 1.50	0.75 ± 0.50[b]	0	2.00 ± 1.83	3.75 ± 1.89	1.89 ± 0.93
4	10 ppm pitavastatin (4/4/5)	0	0	0	0	0	0
5	AOM (4/4/5)	0	0.25 ± 0.50	0	0.25 ± 0.50	0	0
6	2% DSS (4/4/10)	6.00 ± 2.16	3.75 ± 1.71	0	0.25 ± 0.50	0	0
7	None (4/4/5)	0	0	0	0	0	0

Data were from histopathological analysis.
[a]Mean ± SD.–[b]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.05$).

TABLE IV – INCIDENCE OF COLONIC TUMOR AT WEEKS 5, 10 AND 20

Group no.	Treatment	Adenoma			Adenocarcinoma			Total		
		Wk 5	Wk 10	Wk 20	Wk 5	Wk 10	Wk 20	Wk 5	Wk 10	Wk 20
1	AOM/2% DSS (4/4/9)	4/4, 100%	3/4, 75%	9/9, 100%	4/4, 100%	3/4, 75%	9/9, 100%	4/4, 100%	4/4, 100%	9/9, 100%
2	AOM/2% DSS/1 ppm pitavastatin (4/4/10)	4/4, 100%	4/4, 100%	9/10, 90%	3/4, 75%	3/4, 75%	9/10, 90%	4/4, 100%	4/4, 100%	9/10, 90%
3	AOM/2% DSS/10 ppm pitavastatin (4/4/9)	2/4, 50%	4/4, 100%	7/9, 78%	2/4, 50%	4/4, 100%	7/9, 78%	2/4, 50%	4/4, 100%	8/9, 89%
4	10 ppm pitavastatin (4/4/5)	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%
5	AOM (4/4/5)	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%
6	2% DSS (4/4/10)	0/4, 0%	0/4, 0%	0/10, 0%	0/4, 0%	0/4, 0%	0/10, 0%	0/4, 0%	0/4, 0%	0/10, 0%
7	None (4/4/5)	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%	0/4, 0%	0/4, 0%	0/5, 0%

Data were from histopathological analysis.

Statistical analysis

The incidences among the groups were compared using χ^2 test or Fisher's extract probability test (GraphPad Instat version 3.05, GraphPad Software, San Diego, USA). Other measurements expressing mean ± SD were statistically analyzed using Tukey–Kramer multiple comparison post test (GraphPad Instat version 3.05, GraphPad Software). Differences were considered statistically significant at $p < 0.05$.

Results

General observation

The animals that received DSS in their drinking water (Groups 1, 2, 3 and 6) had bloody stool between Weeks 1–3. Also, some mice treated with AOM/DSS with or without pitavastatin (Groups 1, 2 and 3) had bloody stool, and tumors developed in their colon. However, other groups including Group 4 (the pitavastatin alone group) did not have such symptom. Body weights, liver weights, and relative liver weights in all groups at Week 20 are shown in Table I. With regard to the mean body weights, Group 5 (the AOM alone group, 53.3 ± 6.6 g) significantly increased when compared with all other groups. However, the mean liver weight did not significantly differ among the groups, whereas the mean relative liver weight (g liver weight/100 g body weight) of Group 3 (the AOM/DSS/10 ppm pitavastatin group, 5.94 ± 0.64) was significantly greater than that of Group 2 (the AOM/DSS/1 ppm pitavastatin group, 5.15 ± 0.28, $p < 0.05$), and the value of Group 5 (4.68 ± 0.38) was significantly lower than that of Groups 1 (the AOM/DSS group, 5.56 ± 0.44, $p < 0.05$) and 3 (5.94 ± 0.64, $p < 0.01$). As shown in Table I, the mean length of the colon did not significantly differ among the groups.

Incidence and multiplicity of colonic mucosal ulcer and dysplasia

Table II summarizes the incidence of colonic mucosal ulcer (Fig. 3*a*) and colonic dysplasia (Fig. 3*b*) at each time point. The incidence of mucosal ulcer gradually decreased as administration of pitavastatins doses increased at each time-point. On the other hand, the incidence of dysplasia were unaffected. As given in Table III, the multiplicity of mucosal ulcer in Groups 1, 2, 3 and 6 was the highest at Week 5, and then they gradually decreased. At Weeks 5 and 10, the value was decreased by administration of pitavastatin dose-dependently. The multiplicity of mucosal ulcer of Group 3 ($p < 0.05$) was significantly decreased when compared with Group 1. At Week 20, mucosal ulcer was not found in mice of Group 3. Dysplastic crypts were also present in mice given AOM and DSS with or without pitavastatin treatment at Week 5. Colonic dysplasia tended to decrease during the experiment, as did mucosal ulcer. The multiplicities of dysplasia in the mice of Groups 2 and 3 were lower than that of Group 1, but the differences among the groups did not reach statistical significance.

Incidence and multiplicity of large bowel neoplasms

Table IV shows the incidence of colonic tumor at each time-point. It was observed that adenoma (Fig. 3*c*) and adenocarcinoma (Fig. 3*d*) located in the middle and distal colon at each time point. However, treatment with pitavastatin unaffected the incidence of colonic tumor at Weeks 10 and 20. The multiplicities of colonic neoplasms at Weeks 5, 10 and 20 are given in Table V. Colonic adenoma and adenocarcinoma were observed even at Week 5. The multiplicities of adenoma in Groups 2 and 3 were smaller than that of Group 1 at weeks 5 and 20, but the differences were not statistically significant among the groups. As for the

TABLE V – MULTIPLICITIES OF COLONIC TUMOR AT WEEKS 5, 10 AND 20

Group no.	Treatment (no. of mice examined at wk 5/wk 10/wk 20)	Adenoma			Adenocarcinoma			Total		
		Wk 5	Wk 10	Wk 20	Wk 5	Wk 10	Wk 20	Wk 5	Wk 10	Wk 20
1	AOM/2% DSS (4/4/9)	4.00 ± 1.15[a]	2.25 ± 1.71	3.82 ± 1.78	3.00 ± 1.63	5.30 ± 1.30	5.27 ± 3.13	7.00 ± 1.15	7.50 ± 2.38	9.09 ± 3.86
2	AOM/2% DSS/1 ppm pitavastatin (4/4/10)	2.00 ± 0.82	2.25 ± 1.89	2.70 ± 1.42	2.30 ± 1.70	1.50 ± 1.29	1.50 ± 0.97[b]	4.25 ± 2.06	3.75 ± 2.75	4.20 ± 2.10[c]
3	AOM/2% DSS/10 ppm pitavastatin (4/4/9)	1.25 ± 1.50	3.75 ± 0.96	3.00 ± 1.87	1.50 ± 1.90	1.50 ± 1.00	2.00 ± 1.00[c]	2.75 ± 3.20	5.25 ± 1.89	5.00 ± 2.50[d]
4	10 ppm pitavastatin (4/4/5)	0	0	0	0	0	0	0	0	0
5	AOM (4/4/5)	0	0	0	0	0	0	0	0	0
6	2% DSS (4/4/10)	0	0	0	0	0	0	0	0	0
7	None (4/4/5)	0	0	0	0	0	0	0	0	0

All data were from histopathological analysis.
[a]Mean ± SD.—[b,c,d]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ([b]$p < 0.001$, [c]$p < 0.01$, and [d]$p < 0.05$).

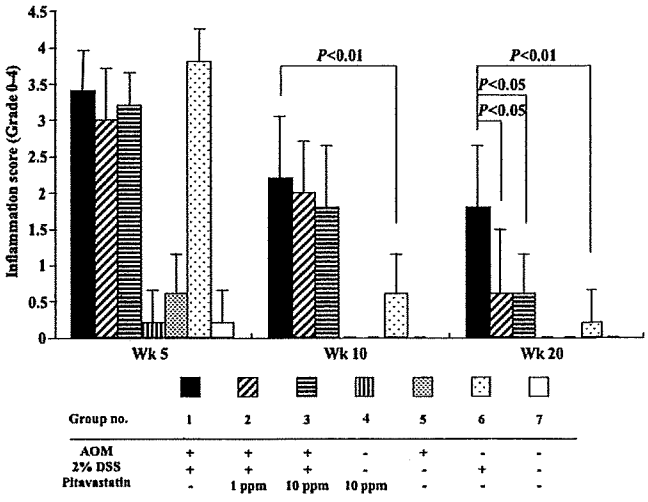

FIGURE 4 – Inflammatory scores in the large bowel of mice in all groups.

multiplicity of adenocarcinoma, the values of Groups 2 and 3 were low without statistical significance when compared to Group 1 at Weeks 5 and 10. However, the values of Groups 2 ($p < 0.001$) and 3 ($p < 0.01$) at Week 20 were significantly smaller than that of Group 1, although the inhibition was not dose-dependent.

Inflammation score in the large bowel

Figure 4 illustrates data on colonic inflammation scores at Weeks 5, 10 and 20. The inflammation scores of Groups 1, 2, 3 and 6 were the greatest at Week 5, and they gradually lowered with time. Colonic inflammation in the mice of Groups 4, 5 and 7, which were not given 2% DSS, were not observed at Weeks 10 and 20, while they had slight colitis at Week 5. At Weeks 5 and 10, the scores in Groups 2 and 3 that were given pitavastatin-containing diets were smaller than that of Group 1, but the differences did not reach the statistical significance. However, their scores were significantly lower than Group 1 at Week 20 (*vs.* Group 2, $p < 0.05$; Group 3, $p < 0.05$; and Group 6, $p < 0.01$).

Immunohistochemical scores for PCNA-, ssDNA- and nitrotyrosine-positive cells in the colonic adenocarcinomas

Scoring data on PCNA- (Fig. 5*a*) and ssDNA- (Fig. 5*b*) in adenocarcinoma cells and nitrotyrosine-positivity (Fig. 5*c*) in colonic mucosa with or without tumors are illustrated in Figure 6. As shown in Figure 6*a*, the mean PCNA-labeling indices of colonic adenocarcinomas developed in Groups 2 ($p < 0.001$) and 3 ($p < 0.001$) were significantly lower than that of Group 1. The mean apoptosis indices of Groups 2 ($p < 0.05$) and 3 ($p < 0.001$), which were measured by ssDNA immunohistochemistry, were significantly greater than that of Group 1, as shown in Figure 6*b*. Immunoreactivity of nitrotyrosine was noted in the adenocarcinoma cells (Fig. 5*c*). The reaction was also observed in the cryptal cells with or without disruption, infiltrated mononuclear inflammatory cells and endothelial cells of the small vessels in the mucosa and submucosa (Fig. 5*c*). The positive reaction was not detected in the colon of mice in Groups 4, 5 and 7. As illustrated in Figure 7, the scores of nitrotyrosine-positivity in Groups 1, 2, 3 and 6 were the greatest at Week 5, and decreased with time. At Week 5, the scores of Groups 2 ($p < 0.001$), 3 ($p < 0.001$) and 6 ($p < 0.05$) were significantly lower than that of Group 1. At Week 10, the scores of Groups 2 ($p < 0.01$), 3 ($p < 0.001$) and 6 ($p < 0.001$) were significantly lower than that of Group 1. Also, the scores of Groups 3 ($p < 0.05$) and 6 ($p < 0.01$) were significantly lower than that of Group 1 at Week 20.

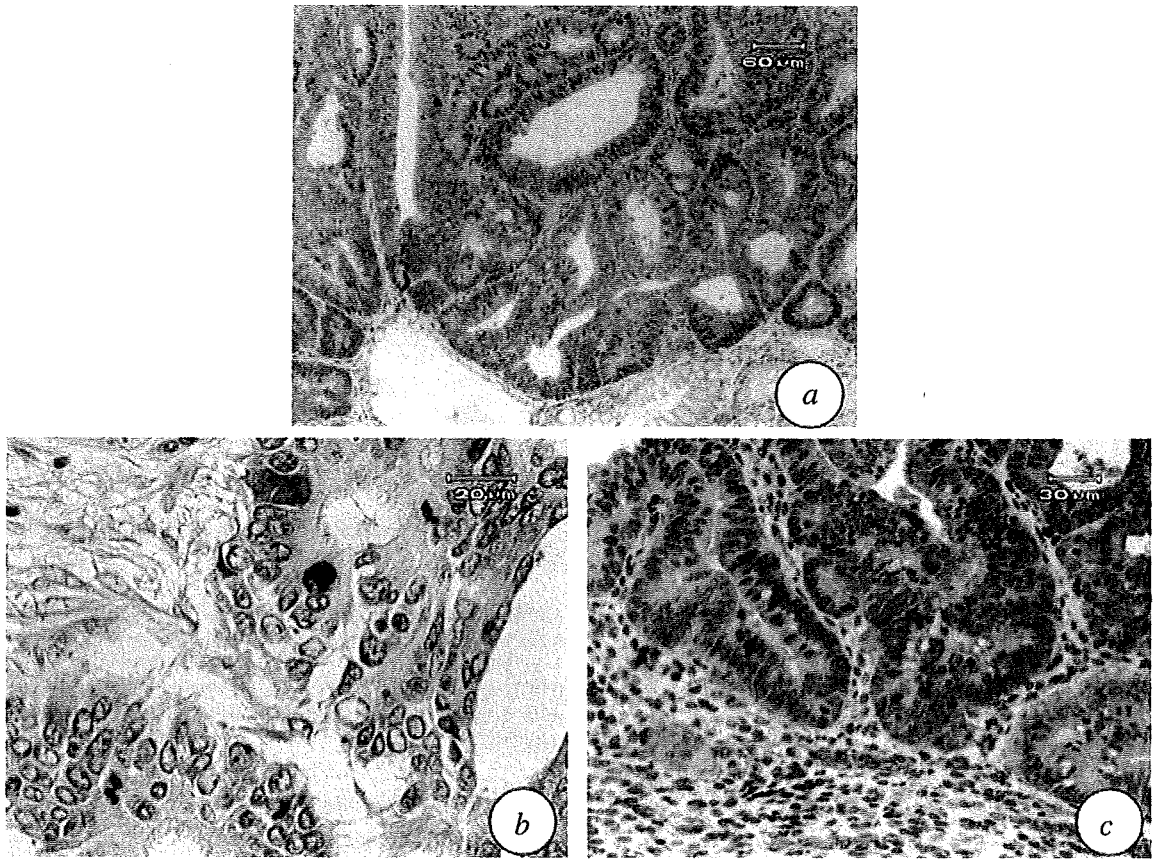

FIGURE 5 – Immunohistochemistry of (*a*) PCNA-labeled nuclei, (*b*) ssDNA-positive nuclei and (*c*) nitrotyrosine-positive cells in adenocarcinomas developed in the colon of a mouse from Group 1. Bars inserted are (a) 60 μm, (b) 20 μm and (c) 30 μm.

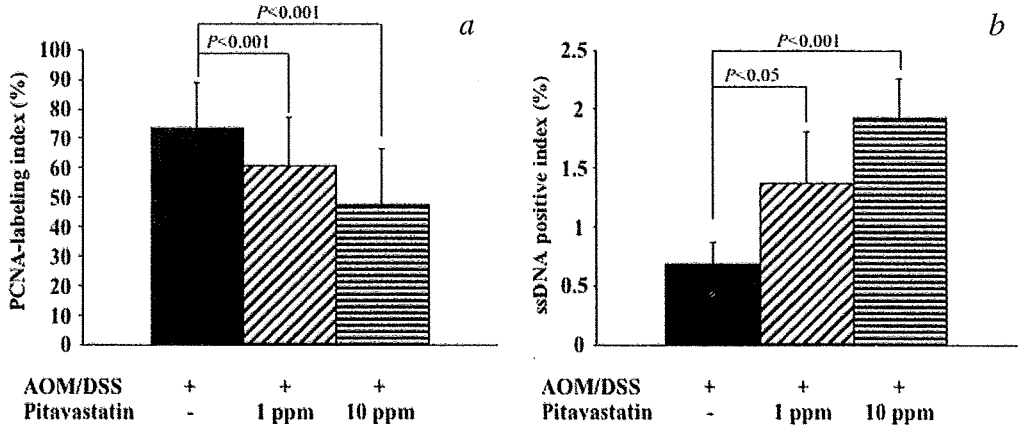

FIGURE 6 – Indices of (*a*) PCNA-labeled nuclei and (*b*) ssDNA-positive nuclei in 5 colonic adenocarcinomas each from Groups 1–3 at Week 20.

Serum levels of TG and total cholesterol

Table VI summarizes data on serum levels of TG and total cholesterol at each time point. The serum TG level of Group 1 (AOM/DSS group) was the greatest among the group at 3 time points. At Week 20, but not at Weeks 5 and 10, the values of Groups 2 ($p < 0.001$) and 3 ($p < 0.001$) were significantly lower than that of Group 1. Similarly, the serum level of total cholesterol of Group 3 ($p < 0.05$) was significantly smaller than that of Group 1, as listed in Table VII.

Discussion

In the current study, we first demonstrated cancer chemopreventive effects of pitavastatin on colitis-related mouse colon carcinogenesis induced by AOM/DSS. Suppressing effects of colitis-related colon carcinogenesis by pitavastatin may be due to reduction of cell proliferation, induction of apoptosis, inhibition of inflammation and suppression of oxidative/nitrosative stress in the colonic malignancy. In the current study, treatment with pitavastatin alone (Group 4) did not affect colonic morphology, including

induction of ulcer and neoplasms. This is important, since a recent case report described the development of UC in a patient who took simvastatin and was fatal.[34]

In the current study, we observed that dietary pitavastatin inhibits the multiplicity, but not the incidence, of colonic adenocarcinomas induced by AOM/DSS. This may be related to weak chemopreventive effects of a low dose of pitavastatin. Also, there was no dose-response of the inhibition, although data on the indices of PCNA and ssDNA may suggest that pitavastatin affects dose-dependently proliferation and apoptosis in adenocarcinoma cells. Since only 2 doses (1 and 10 ppm in diet) of pitavastatin were used for assessing chemopreventive ability of the drug against AOM/DSS-induced mouse colon carcinogenesis in this study, additional doses (>10 ppm in diet) must be investigated to determine the dose-dependent efficacy of pitavastatin in suppressing AOM/DSS-induced colon carcinogenesis. As for colonic adenoma, the incidence did not significantly alter at 3 time points (Weeks 5, 10 and 20). The multiplicity of Group 2 was increased with time, but the increase was insignificant. The findings may suggest that a high dose (10 ppm) of pitavastatin is able to inhibit progress from adenoma to adenocarcinoma.

While statins are primarily known as drugs for the treatment of hypercholesterolemia because of their potency of reduction in LDL-cholesterol level by competitively inhibiting HMG-CoA reductase that is a rate-limiting enzyme in the synthesis of mevalonate, they have pleiotropic distinct effects on process such as angiogenesis[35] and inflammation.[36,37] Thus, statins affect a number of novel molecular targets and complex signaling pathways. Certain statins (simvastatin and rosuvastatin) are able to exert anti-inflammatory action in DSS-induced acute or chronic murine colitis model.[38,39] A lipophilic statin pitavastain also possesses multiple biological function[25] and anti-inflammatory action.[29,30] Pitavastatin is recently reported to down-regulate chemokines[40] that are involved in IBD pathogenesis.[41] Also, a low dose of pitavastatin can affect PI3K-AKT pathway,[42] which plays a critical role in the balance between cell survival and apoptosis, the inflammatory response by activating chemokine receptors and promoting inflammatory cell migration and the human cancer development,[43–45] including colon cancer.[46] In the current study, the treatment with pitavastatin in diet significantly lowered colonic inflammation induced by DSS, as revealed by histopathology (number of mucosal ulcer and inflammation scores). As observed in the colonic mucosa of UC patients, where colonic mucosal damage is associated with increased production of nitric oxide (NO) through the inducible nitric oxide synthase (iNOS) pathway,[47] the numbers of cryptal, infiltrated inflammatory, endothelial and tumor cells positive for nitrotyrosine, being a good biomarker for nitrosative stress,[48] were increased in the inflamed colon induced by DSS in this study. Pitavastatin treatment significantly lowered the nitrotyrosine-positive immunohistochemical score in conjunction with reduction in the number of mucosal ulcer and inflammatory score. iNOS is reported to be over-expressed in colonic tumors of humans[49] and chemically induced colonic tumors.[50] Although there are no reports that pitavastatin affects iNOs expression in inflamed tissues and neoplasms, our findings may suggest the possible effects of pitavastatin on iNOS expression. Activated nuclear factor-kappa B (NF-κB), which is a key player in inflammatory processes in the tissues,[51,52] is observed in different cancer cell lines and primary malignant tissue samples.[53] Recently, Lee *et al.*[38] demonstrated that simvastatin inhibits proinflammatory gene expression by blocking NF-κB signaling in intestinal epithelial cells, and attenuates DSS-induced acute murine colitis. Wang *et al.*[54] have also found that pitavastatin inhibits NF-κB activation and decreases IL-6 production induced by tumor necrosis factor-α in human hepatocellular carcinoma cells. NF-κB activation also plays an important role in enhancing IL-6 and IL-8 in human colon cancer cells.[55] Although we did not examine mRNA expression of NF-κB and cytokines in this study, it is possible that paitavastatin affects the expression in the inflamed mouse colon. The anti-inflammatory and antioxidative/nitrosative potential of pitavastatin is possibly related to prenylation of certain proteins that are involved in inflammatory processes,[56,57] but not its effect on HMG-CoA enzyme, as is the case of other statins.[17,58] The findings reported by others and those described here, thus, may suggest the potential use of statins, including pitavastatin as an anti-inflammatory drug for the treatment of IBD.

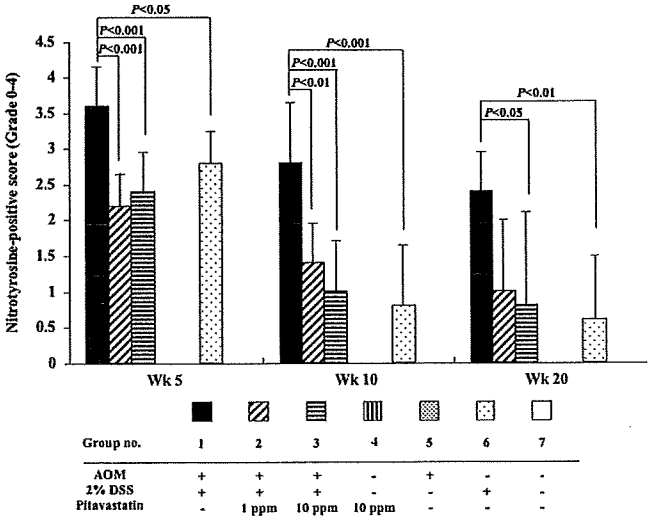

FIGURE 7 – Nitrotyrosine-positive indices in the colonic mucosa with or without tumors from all the mice of each sacrifice time point (4 mice each from all groups at Week 5; 4 mice each from all groups at Week 10; and 9 mice each of Groups 1 and 3, 10 mice each of Groups 2 and 6, and 5 mice each of Groups 4, 5 and 7 at Week 20).

Other interesting findings in the current study are that administration of pitavastatin induced apoptosis in the colonic epithelial malignancies. There are no reports describing apoptosis-inducing effects of pitavastatin on tumor cells, although certain statins possess proapoptotic properties in a variety of tumor cell lines.[59–62]

TABLE VI – SERUM TRIGLYCERIDE (MG/DL) AT WEEKS 5, 10 AND 20

Group no.	Treatment	Wk 5	Wk 10	Wk 20
1	AOM/2% DSS	134.8 ± 63.5[a] (5)	174.6 ± 96.7 (5)	159.0 ± 59.7 (9)
2	AOM/2% DSS/1 ppm pitavastatin	79.4 ± 27.5 (5)	117.4 ± 21.7 (5)	64.4 ± 16.8[b] (7)
3	AOM/2% DSS/10 ppm pitavastatin	67.2 ± 26.8 (5)	84.2 ± 28.0 (5)	61.0 ± 27.5[b] (7)
4	10 ppm pitavastatin	77.8 ± 36.8 (5)	67.2 ± 13.3 (5)	59.0 ± 23.4 (5)
5	AOM	126.0 ± 51.2 (5)	92.0 ± 35.9 (5)	94.8 ± 34.0 (5)
6	2% DSS	70.4 ± 33.4 (5)	105.2 ± 24.8 (5)	79.3 ± 37.9 (7)
7	None	105.2 ± 38.1 (5)	54.0 ± 15.3 (5)	54.5 ± 16.0 (4)

Numbers of parentheses are numbers of mice examined.
[a]Mean ± SD.–[b]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.001$).

TABLE VII – SERUM TOTAL CHOLESTEROL (MG/DL) AT WEEKS 5, 10 AND 20

Group no.	Treatment	Wk 5	Wk 10	Wk 20
1	AOM/2% DSS	137.2 ± 10.0[a] (5)	137.4 ± 22.7 (5)	152.8 ± 43.7 (9)
2	AOM/2% DSS/1 ppm pitavastatin	127.6 ± 14.8 (5)	119.1 ± 20.9 (5)	114.9 ± 18.2 (7)
3	AOM/2% DSS/10 ppm pitavastatin	156.4 ± 26.2 (5)	105.2 ± 10.5 (5)	105.1 ± 23.5[b] (7)
4	10 ppm pitavastatin	117.2 ± 19.1 (5)	109.0 ± 10.7 (5)	106.6 ± 7.6 (5)
5	AOM	134.8 ± 20.6 (5)	146.4 ± 29.2 (5)	161.4 ± 33.3 (5)
6	2% DSS	151.2 ± 28.2 (5)	137.6 ± 35.4 (5)	119.1 ± 20.3 (7)
7	None	151.8 ± 14.6 (5)	140.6 ± 18.4 (5)	137.5 ± 25.1 (4)

Numbers of parentheses are numbers of mice examined.
[a]Mean ± SD.–[b]Significantly different from Group 1 by Tukey–Kramer multiple comparison post test ($p < 0.05$).

Lipophilic statins are reported to induce apoptosis in malignant cells. For example, Agarwal *et al.*[59] reported that lovastatin induces apoptosis with differing sensitivity in a variety of colon cancer cell lines (SW480, HCT 116, LoVo and HT29). They also found that lovastatin treatment results in decreased expression of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic protein Bax. There are some reports describing the comparison of apoptosis inducing ability between lipophilic and hydrophilic statins in tumor[60] and nontumor cells.[63,64] These reports suggested that lipophilic statins are more effective for inducing apoptosis when compared to hydrophilic statins. As to antiproliferative action of statins, the effect of lovastatin on prostate cancer cells is stronger than that of a hydrophilic statin, pravastatin.[65] Thus, the lipophilic property of pitavastatin may be related to the apoptosis induction and inhibition of proliferation in adenocarcinomas observed in this study.

Statins, including pitavastatin, are drugs that primarily affect LDL-cholesterol levels in plasma through the induction of the hepatic LDL receptor.[66] In this experiment, pitavastatin treatment effectively lowered serum total cholesterol level at Week 20. In addition, administration of pitavastatin significantly decreased serum TG level that was 3-fold increased by AOM/DSS exposure at Week 20. Hypertriglyceridemia is a risk for human CRC development.[67,68] Also, hyperlipidemia is a relatively frequent complication in patients with familial adenomatous polyposis patients.[69] In this context, a recent report[70] that lipoprotein lipase gene polymorphism influences lipid metabolism in UC patients and age of onset of UC is of interest.

A growing body of literature has emerged on the prevention of CRC in patients with long-standing CD and UC.[71,72] However, the data are not definitive and consist almost exclusively of retrospective case–control and cohort studies rather than the more rigorous prospective multiple randomized controlled trials.[31] Although the data on statins use are still too limited to endorse its use for the prevention of colitis-related CRC, further studies with statins need to be performed to develop an optimal strategy for the reduction of cancer risk in IBD patients. While most statins are metabolized in part by one or more hepatic cytochrome P450 enzymes (mainly CYP3A4), leading to an increased potential for drug interactions and problems with certain foods, such as grapefruit juice, pitavastatin appears to be metabolized by a substrate of CYP2C9.[25] This property may prove beneficial for the long-term use of the drug in clinic.

In conclusion, our current findings that a lipophilic statin pitavastatin was effective for inhibiting colitis-related mouse colon carcinogenesis through modulating the cell proliferation, mucosal inflammation and oxidative/nitrosative stress in the target tissue suggest possible application of pitavastatin in suppressing colon carcinogenesis in the inflamed colon of patients with IBD. Further studies on detailed mechanisms of the action involved are underway in our laboratory using microarray and proteomics techniques.

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A novel *ras*H2 mouse carcinogenesis model that is highly susceptible to 4-NQO-induced tongue and esophageal carcinogenesis is useful for preclinical chemoprevention studies

Shingo Miyamoto, Yumiko Yasui[1], Mihye Kim, Shigeyuki Sugie[1], Akira Murakami, Rikako Ishigamori-Suzuki[1] and Takuji Tanaka*

Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan and [1]Department of Oncologic Pathology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

*To whom correspondence should be addressed. Tel: +81 76 218 8116; Fax: +81 76 286 6926;
Email: takutt@kanazawa-med.ac.jp

We investigated the susceptibility of 4-nitroquinoline 1-oxide (4-NQO)-induced tongue carcinogenesis in male CB6F1-Tg-*ras*H2 @Jcl mice (Tg mice). The Tg mice were administered 4-NQO (20 p.p.m. in drinking water) for 2, 4, 6 or 8 weeks, and thereafter they were untreated up to week 24. At week 24, a higher incidence (80%) of tongue neoplasm with dysplasia was noted in the mice that received 4-NQO for 8 weeks in comparison with the other groups (20% incidence for each) treated with 4-NQO for 2, 4 and 6 weeks. Esophageal tumors also developed in the Tg mice were 4-NQO. Immunohistochemical observation revealed that the EP receptors, especially EP_1 and EP_2, expressed in the tongue and esophageal lesions induced by 4-NQO, thus suggesting the involvement of prostaglandin (PG) E_2 and $EP_{1,2}$ receptors in the tongue and esophageal carcinogenesis. Using this animal model, we investigated the potential chemopreventive ability of pitavastatin (1, 5 and 10 p.p.m. in diet for 15 weeks), starting 1 week after the cessation of 4-NQO-exposure (20 p.p.m. in drinking water for 8 weeks). Dietary pitavastatin at 10 p.p.m. significantly reduced the incidence and multiplicity of the tongue, but not esophageal neoplasms by the modulation of prostaglandin E2 biosynthesis, EP_1 and EP_2 expression and proliferation. Our results thus suggest that a *ras*H2 mouse model of 4-NQO-induced tongue and esophageal carcinogenesis can be utilized for investigating the pathogenesis of cancer development in these tissues and may well prove to be useful for identifying candidate cancer chemopreventive agents for the upper digestive organs.

Introduction

Oral cancer is the 11th most common human neoplasm and accounts for 3% of all newly diagnosed cancers (1). While this epithelial malignancy mainly developed in the elderly in the past, an increased number of young patients with oral malignancy being seen at present in Europe, North America and India are increased today (2,3). Such prevalence is largely associated with the habit of using chewing tobacco and other related products. More than 90% of oral cancers are histopathologically squamous cell carcinomas (SCCs) (4), with >300 000 new cases being diagnosed every year worldwide (5). Despite efforts to improve the overall outcome, the survival rates of oral cancer patients have not changed for the last 20 years. Since ~50 to 70% of patients die within 5 years due to local recurrence, invasion or metastasis in the esophagus and/or lung, or second primary cancers, generally elsewhere in the oral cavity ('field cancerization' theory) (6–8), the prognosis is poor. The detection of many cancers including oral cancer is often too late for successful intervention. Therefore, if appropriate biological markers can identify pre-malignant lesions, then we can detect them and prevent cancer development before they progress to malignancies by the use of chemoprevention modalities or other therapeutics (9,10). The incidence rates for one of the oral pre-malignant lesions, leukoplakia with dysplastic nature, in the Japanese are somewhat higherthan those reported from India, since the risk habits of the two countries are both markedly different (11).

The development of oral cancer is a multistep process, which includes hyperplasia, dysplasia and finally neoplasm (benign and malignant) (10). During these steps, the accumulation of genetic alterations, including chromosomal aberrations, DNA mutations, amplifications or deletions and/or epigenetic alterations (methylation) occur (12–15). These events are often influenced by exposure to environmental agents. These include tobacco smoke, alcohol beverages and viruses as the major risk factors (10). In patients, the analysis of these events during the multistep process is hampered by the unavailability of biopsies obtained at all stages (namely hyperplasia, dysplasia and neoplasm) of carcinogenesis. However, animal models of oral carcinogenesis allow the reproducible isolation of all stages, including normal tissues, which are then amenable to pathological, genetic and biochemical analyses. Thus, appropriate animal models are essential for investigating the transition of oral squamous epithelium from normal through dysplastic states and ultimately into SCC. There are several animal models for oral carcinogenesis (16). Among them, the systemic application of 4-nitroquinoline 1-oxide (4-NQO) via drinking water is easily able to induce tongue tumors in rats (10,17) and mice (18). Recent reviews (5,19) have concluded that the rat 4-NQO-induced tongue carcinogenesis is the main model for simulating the process of oral carcinogenesis in humans with a fair degree of reliability. However, in contrast to rats (10), oral and esophageal tumors develop in mice that receive 4-NQO in drinking water (18). This suggests that a mouse model initiated with 4-NQO is useful for investigating field cancerization in the regions (8).

H-*ras* mutations are implicated in human and murine oral carcinogenesis (20–22). *Ras* mutations are observed in oral cancer with different frequencies in different countries (20,23). Our recent findings suggest that human c-Ha-*ras* proto-oncogene-carrying transgenic rats are highly susceptible to a water-soluble carcinogen 4-NQO in their tongue (24), thus suggesting an excellent rat model for investigating oral cancer development and treatment/chemoprevention of this malignancy. Few studies, nevertheless, have addressed the aspect of *ras* activation, while considering its possible role in mouse oral carcinogenesis. CB6F1-Tg *ras*H2@Jcl mice (Tg mice) were developed by Saitoh *et al.* (25) to evaluate the association of chemically induced transgene expression and tumor induction (26,27). Three copies of the human transgene were integrated into the mouse genome in a tandem array through pronuclear injection. The Tg mice are hemizygous for the human c-Ha-*ras* transgene under control of its endogenous promoter and enhancer sequences. Expression of the transgenic protein is observed in normal tissues and increased ~2-fold in chemically induced tumors (28). Mutation of the endogenous mouse ras genes or of the transgene is infrequent and unpredictable.

Prostaglandins (PGs) are generated via the cyclooxygenase (COX)-1 and -2 and are known to be elevated in the rat tongue following 4-NQO exposure (29). COX-1 and COX-2 both catalyze the first reaction in the conversion of arachidonic acid into PGs, of which prostaglandin E_2 (PGE_2) is the major product found in the rat tongue exposed to 4-NQO (29). COX-1 is the constitutively expressed isoform, and COX-2 is the inducible isoform (30). Although a number of studies indicate that the inhibition of PGE_2 biosynthesis through COX-2 expression contributes to the suppression of cancer development in a variety of tissues (31), including tongue cancer (24,32). COX-2 deregulation is reported in smokeless tobacco-related oral

Abbreviations: COX, cyclooxygenase; PAP, squamous cell papilloma; PBS, phosphate buffer saline; PCNA, proliferative cell nuclear antigen; PG, prostaglandin; PGE_2, prostaglandin E_2; SCC, squamous cell carcinoma; 4-NQO, 4-nitroquinoline 1-oxide.

