Citrus Compounds Inhibit Inflammation- and Obesity-Related Colon Carcinogenesis in Mice

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Dietary polyphenols are important potential chemopreventive natural agents. Other agents, such as citrus compounds, are also candidates for cancer chemopreventives. They act on multiple key elements in signal transduction pathways related to cellular proliferation, differentiation, apoptosis, inflammation, and obesity. This short review article provides our findings of preclinical studies on potential chemopreventive activities of dietary citrus compounds, auraptene, collinin, and citrus unshiu segment membrane (CUSM), using clitis- and obesity-related colon tumorigenesis models. Dietary feeding with auraptene and collinin at dose levels of 0.01% and 0.05% significantly lowered the incidence (50–60% reduction) and multiplicity (67–80% reduction) of colonic adenocarcinomas induced by azoxymethane [AOM, single intraperitoneal injection of 10 mg/kg body weight (bw)] and dextran sodium sulfate (1% in drinking water). Anti-inflammatory potency of aurapene and collinin may contribute to the effects. Administration with CUSM at 3 doses in diet significantly inhibited development of aberrant crypt foci induced by 5 weekly subcutaneous injections of AOM (15 mg/kg bw) in male db/db mice: 53% inhibition by 0.02% CUSM, 54% inhibition by 0.1% CUSM, and 59% inhibition by 0.5% CUSM. CUSM treatment also decreased serum level of triglycerides. Our findings suggest that certain citrus materials are capable of inhibiting clitis- and obesity-related colon carcinogenesis.

INTRODUCTION

Colorectal cancer (CRC) has increased in Asia owing to the changes in lifestyle such as the dietary habit of increased meat consumption (1,2). Inflammation and obesity are risk for chronic diseases including cancer development (3–5). In fact, CRC is one of the most serious complications of inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn’s disease (6). Long-term UC patients have high risk of developing colorectal cancer compared with the general population (7). Obesity raises the risk for a variety of disease (8). Numerous epidemiological results suggest that obesity is a risk factor for colon cancer (9,10). There are several studies that have investigated the possible mechanism for this (11,12). Obesity is a complex, heterogeneous, and multifactorial syndrome resulting from both genetic susceptibility and environmental factors (13). Besides obesity, it is well known that several factors, including a high-fat and low-fiber diet (14), low physical activity (15), IBD (16) or hereditary disorders such as familial adenomatous polyposis and nonpolyposis syndrome (17), increase the risk for development of CRC.

Fighting IBD- and obesity-related CRC as well as sporadic CRC by cancer chemoprevention strategy is important to reduce the risk, and thus primary prevention of CRC in IBD and/or obese people has recently been receiving more attention. There are several natural compounds that are able to inhibit CRC development in rodents, and thus they are candidates for cancer chemopreventive agents (18–21). For fighting this malignancy in inflamed colon and colon of obese people, we tried to find natural compounds that are capable of effectively inhibiting colon carcinogenesis in a series of preclinical studies (22–24). In this article, we introduce our findings for pushing candidate materials into clinical trials.

METHODS: PRECLINICAL EXPERIMENTS

Preclinical Chemopreventive Study 1: Citrus Auraptene and Related Compound Collinin Inhibit Azoxymethane (AOM)/Dextran Sodium Sulfate (DSS)-Induced Colitis-Related Colon Carcinogenesis in Mice

Previous experimental and epidemiological investigations suggest that several agents, such as folic acid (25), conjugated linoleic acid (26), ursodeoxycholic acid (27), 5-aminosaliclyc acid (28), and aspirin, may reduce the occurrence of CRC in patients with IBD (29,30). Consistent with these data, several nonsteroidal anti-inflammatory drugs (NSAIDs), including cyclooxygenase (COX)-2 inhibitors, suppressed the development of chemically induced CRC in rats (31) and intestinal polyps (tubular adenomas) in Min mice with a nonsense mutation of the adenomatous polyposis coli (APC) gene (32). In addition, clinical trials demonstrated that intake of sulindac causes regression of adenomas in patients with familial adenomatous polyposis (33). Epidemiological studies indicate an inverse correlation
between the intake of fruits/vegetables and human CRC (34). Thus, primary prevention including chemoprevention using the active compounds in fruits/vegetables is also important for reducing the risk of this malignancy. Citrus fruit contains several chemopreventive compounds against CRC (20,35–37). Prenyloxy coumarins including auraptene (Fig. 1a) and collinin (Fig. 1b) are candidates of such chemopreventers. They are secondary metabolites mainly found in plants belonging to the families of Rutaceae and Umbelliferae. Several of these coumarins were shown to possess valuable pharmacological properties. These compounds were reported to have anti-inflammatory activity (38). Auraptene significantly attenuated the lipopolysaccharide (LPS)-induced protein expression of inducible nitric oxide synthase (iNOS) and COX-2, with decreases in production of nitric anion and prostaglandin E2 (PGE2), and yet suppressed the release of tumor necrosis factor (TNF)-α and IκB degradation (39,40). Furthermore, auraptene and collinin also cause complete inhibition of platelet aggregation induced by arachidonic acid and platelet activated factor in vitro (41). We have previously found that a citrus auraptene suppresses chemically induced carcinogenesis in a variety of tissues of rodents (20,42), as shown in Fig. 1. We also demonstrated that dietary administration of a COX-2 inhibitor and peroxisome proliferator-activated receptor ligands suppressed colitis-related colon carcinogenesis using our mouse colon carcinogenesis model (43).

In this experiment (Study 1), to determine the effects of auraptene and collinin in inflammation-related colon tumorigenesis, we utilized a novel colitis-related mouse CRC model using a colon carcinogen AOM and DSS in which large bowel adenocarcinomas develop within a short-term period and their histology and biological alteration resemble those found in humans (44). This animal model indicates that in the large bowel, inflammation induced by DSS strongly promotes the development of epithelial malignant neoplasia (45–50). Oxidative/nitrosative stress caused by DSS exposure may contribute the development of high incidence of colonic adenocarcinomas with certain genetic alterations (45, 6), as shown in Table 1 and Figs. 2 and 3.

A total of 75 male ICR mice aged 5 wk (Charles River Japan Inc., Tokyo, Japan) were divided into 10 experimental and control groups. The study was approved by the Institutional Animal Care Guidelines, and the experiment complied with the International Guiding Principles for Biomedical Research Involving Animals established by the Council for International Organization of Medical Sciences (CIOMS). Mice in Groups 1 through 5 were given a single intraperitoneal injection of AOM (10 mg/kg body weight, Sigma Chemical Co., St. Louis, MO). Starting 1 wk after the injection, animals were administered to 1% (wt/vol) DSS (molecular weight 36,000–50,000; ICN Biochemicals, Inc., Aurora, OH) in drinking water for 7 days and then followed without any further treatment for 15 wk. Mice of Group 1 were maintained on the basal diet Charles River Formula (CRF)-1 (Oriental Yeast Co., Ltd., Tokyo, Japan) throughout the study. CRF-1 consisted of 5.7% fat, 22.4% protein, 6.6% minerals, 3.1% fiber, and 62.3% carbohydrate and others (≈3.59 kcal/g). The major fatty acids present in CRF-1 were linoleic acid, oleic acid, and palmitic acid. Mice in Groups 2 through 5 were given 0.01% auraptene in diet (Group 2),
TABLE 1
Sequential observation of AOM/DSS-induced mouse colon tumorigenesis*.

<table>
<thead>
<tr>
<th>Experimental Weeks</th>
<th>Incidence (multiplicity) of colonic adenocarcinomas</th>
<th>Immunohistochemical nitrotyrosine-positive scores</th>
</tr>
</thead>
<tbody>
<tr>
<td>Week 2</td>
<td>0 (0)</td>
<td>7.32 ± 1.35 ± 0.40</td>
</tr>
<tr>
<td>Week 3</td>
<td>0 (0)</td>
<td>5.22 ± 0.40</td>
</tr>
<tr>
<td>Week 4</td>
<td>40%</td>
<td>5.40 ± 1.32</td>
</tr>
<tr>
<td>Week 5</td>
<td>60%</td>
<td>3.27 ± 0.55</td>
</tr>
<tr>
<td>Week 6</td>
<td>100%</td>
<td>2.09 ± 0.55</td>
</tr>
<tr>
<td>Week 9</td>
<td>100%</td>
<td>2.87 ± 0.62</td>
</tr>
<tr>
<td>Week 12</td>
<td>100%</td>
<td>2.82 ± 0.64</td>
</tr>
<tr>
<td>Week 14</td>
<td>100%</td>
<td>2.75 ± 0.25</td>
</tr>
</tbody>
</table>

**Abbreviations are as follows:** AOM, azoxymethane; DSS, dextran sodium sulfate.

FIG. 2. Experimental protocol of our mouse model for inflammation-related mouse colon carcinogenesis. Incidence and multiplicity of colonic adenocarcinomas in mice that were initiated with azoxymethane (AOM), 1,2-dimethylhydrazine (DMH), or 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhiP) and promoted by dextran sodium sulfate (DSS) and mutations in exon 3 of beta-catenin in induced epithelial malignancies are shown.

FIG. 3. Effects of dose of dextran sodium sulfate (DSS) on azoxymethane (AOM)/DSS-induced mouse colonic carcinogenesis. Treatment with 1% and 2% DSS in drinking water after the initiation with AOM resulted in occurrence of colonic adenocarcinomas. The incidence (a) and multiplicity (b) of colonic adenocarcinomas in male ICR mice given 2% DSS are greater than that of mice treated with 1% DSS.
0.05% auraptene in diet (Group 3), 0.01% collinin in diet (Group 4), or 0.05% collinin in diet (Group 5), respectively, for 17 wk, starting 1 wk after the stop of DSS administration. Group 6 was given a single dose of AOM. Group 7 was given 1% DSS for 7 days. Animals in Groups 8 and 9 were given the diets containing 0.05% auraptene and 0.05% collinin alone, respectively. Group 10 consisted of untreated mice. Auraptene (99.6% purity) (51) and collinin (99.8% purity) (38) were synthesized as described previously. All animals were sacrificed at the end of the study (Week 20) to determine the incidence and multiplicity of colonic tumors and dysplastic crypts. In addition to histopathology, immunohistochemistry for proliferating cell nuclear antigen (PCNA), apoptotic nuclei, COX-2, iNOS, single stranded DNA (ssDNA) (52), and nitrotyrosine was done to determine the effects of dietary auraptene and collinin on their expression in the colonic epithelial malignancies. At Week 12, messenger RNA (mRNA) expression of COX-2, iNOS, interleukin (IL)-1β, and TNF-α was also determined in the nontumorous colonic mucosa of mice in Groups 1, 3, and 5.

At sacrifice (Week 20), nodular or polypoid colonic tumors were observed in the middle and distal colon of mice in Groups 1 through 5. Histopathologically, AOM/DSS-treated mice showed dysplasia, adenoma, and adenocarcinoma. The tumors were histologically diagnosed as tubular adenoma or adenocarcinoma. Animals belonging to Groups 6–10 did not have large bowel neoplasms in any organs examined, including the colon. Group 1 (AOM/DSS) induced 100% incidence of colon adenocarcinomas with a multiplicity of 3.00 ± 1.41. The incidences of colorectal adenocarcinomas in Groups 2 (AOM/DSS/0.01% auraptene), 3 (AOM/DSS/0.05% auraptene), 4 (AOM/DSS/0.01% collinin), and 5 (AOM/DSS/0.05% collinin) were significantly smaller than that of Group 1 (P < 0.05). The multiplicity of colon adenocarcinomas in Groups 2 through 5 were also significantly lower than that of Group 1 (P < 0.05). Colitis was present with or without colonic dysplasia in the middle or distal colon of mice treated with DSS. Colonic inflammation scores in Groups 3 and 5 were significantly decreased when compared with that in Group 1 (P < 0.05). The PCNA-labeling indexes of colonic adenocarcinomas developed in Groups 2–5 were significantly smaller than Group 1 (P < 0.05), and the apoptotic index measured by ssDNA immunohistochemistry in Groups 2, 4, and 5 was significantly greater than Group 1 (P < 0.05). COX-2 expression scores of colonic adenocarcinomas in Groups 2, 3, and 5 and that of iNOS in Groups 2–5 were significantly decreased when compared with that in Group 1 (P < 0.05). The nitrotyrosine-positive scores of Groups 3 and 5 were significantly higher than that of Group 1 (P < 0.05). The scores of Groups 2 and 4 were also lower than that of Group 1, but the differences were insignificant. mRNA expression of the nontumorous colonic mucosa of Groups 3 and 5 were significantly lower than that of Group 1 (P < 0.05), but iNOS expression was comparable among Groups 1, 3, and 5 (Fig. 4).

Preclinical Chemopreventive Study 2: Citrus Unshiu Segment Membranes (CUSM) Suppresses Development of Preneoplastic Lesions in db/db Mice Treated With AOM

The modern Western lifestyle, such as 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 CRC (8), which is 1 of the major causes of morbidity and mortality in the Western world (1). This malignancy has also increased in Asia owing to the changes in lifestyle such as the dietary habit of increased...
meat consumption (1,2). Several prospective and case-control studies have addressed the relationship between obesity/diabetes and CRC (8,53,54).

Certain food components are known to exert a cancer chemopreventive activity against CRC development (55). However, few studies have so far been performed on the preventive effect of food components on obesity- and diabetes-related colon carcinogenesis (56,57). We recently have made CUSM that are rich in soluble and insoluble fiber and separate the juice vesicles from Satsuma mandarin (Citrus unshiu Marc.). Mandarin orange fruit constitutes 9 to 13 segments (juice sacs) that contain juice vesicles, and a membrane that wraps the segment is called the “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 and auraptene. Citrus fibers and flavonoids have been reported to inhibit colon carcinogenesis in rodents (20,58,59). 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 biscuits (60). Supplementation with flavonoids (hesperidin or naringin) improves the hyperglycemia in db/db mice (61). Although the biological activity of CUSM has yet to be elucidated, we suspected that CUSM might have a preventive effect on obesity- and diabetes-related colon carcinogenesis.

C57BL/KsJ-db/db (db/db) mice are used as a genetically altered animal model with genotypes of obesity and diabetes mellitus (62). A disruption of the leptin receptor [obese mutation (Ob)-R] gene in these mice leads to an overexpression of leptin in the adipose tissue and a concomitantly high serum concentration of leptin (63,64). The synthesis of leptin in adipocytes is influenced by insulin (65), TNF-α (66), glucocorticoids (67), reproductive hormones (68), and prostaglandins (69) that may be involved in the neoplastic processes (70). In addition, leptin can act as a growth factor in colonic epithelial cells (71) whereas modulating the proliferation of colonic cryptal cells (72). In contrast, more leptin in the blood clearly decreased colon carcinogenesis (73,74). Thus, leptin might be 1 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.

In this study (Study 2), we determined the possible modulatory effects of dietary CUSM on the occurrence of AOM-induced aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), which are putative precursor lesions for colonic adenocarcinoma (49,75), in db/db, db/+ , and +/- male mice. A total of 36 homozygous db/db, 40 heterozygous db/+ , and 40 littermate controls (+/+) male mice aged 4 wk (Japan SLC, Inc., Shizuoka, Japan) were divided into 4 groups, respectively. The study was approved by the Institutional Animal Care Guidelines, and the experiment complied with the International Guiding Principles for Biomedical Research Involving Animals established by the CIOMS. Animals were free access to drinking water and a basal diet, MF (Oriental Yeast Co., Ltd.). At 5 wk of age, all mice were subcutaneously injected with AOM (15 mg/kg body weight) once a week for 5 wk. Group 1 was fed the basal diet, MF (Oriental Yeast Co., Ltd.), 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 wk, starting 1 wk after the last injection of AOM. The experiment was terminated 12 wk after the start. Powdered CUSM was obtained from 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-sucrose); 2.2 g hesperidin; and 9.7 g others that include flavonoids, carotenoids, and unknown components. At the termination of the study (Week 12), all mice were sacrificed by an overdose of ether to analyze the number of ACF and BCAC. 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 histopathological examination. The stomach, liver, and intestine were fixed in formalin and embedded in paraffin, and then a total of 36 µm sections were made for histopathology (H & E stain) and immunohistochemistry. The liver, kidney, and intestine were subjected to hematoxylin and eosin (H & E) staining for histopathology and β-catenin immunohistochemistry to count the number of colonic BCAC (77,78), and others were used for Ob-R, insulin-like growth factor-1 receptor (IGF-1R), and TNF-α staining for histopathology (H & E stain) and morphometric analysis of liver fibrosis (Sirius-red) and fatty change (H & E stain) using an image analysis software NIH Image v.1.63. 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.

At the end of the study (Week 12), all the mice that received AOM developed colonic ACF and BCAC. Regarding the number of ACF or BCAC in the AOM alone groups, the mean number of db/db mice (147 ± 23, P < 0.05) was significantly higher than that of db/+ (77 ± 19) or +/- mice (69 ± 12). In comparison to the AOM alone group, the dietary administration with CUSM significantly reduced the number of ACF in all the genotypes (P < 0.05): db/db mice, 53% reduction by 0.02% CUSM, 54% reduction by 0.1% CUSM, and 59% reduction by 0.5% CUSM; db/+ mice, 48% reduction by 0.1% CUSM and 38% reduction by 0.5% CUSM; and +/- mice, 45% reduction rate.
by 0.1% CUSM and 62% reduction by 0.5% CUSM. In addition, the percentages of ACF consisting of more than 4 aberrant crypts (ACs) in all the CUSM-feeding groups in the db/db mice (36% reduction by 0.02% CUSM, 30% reduction by 0.1% CUSM, and 47% reduction by 0.5% CUSM) were significantly smaller ($P<0.05$) than that of AOM alone group ($77 \pm 11$). Dietary administration with CUSM reduced the percentages of ACF consisting of more than 4 ACs in the +/+ and +/+ mice; the reduction rates were insignificant. BCAC 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 +/+ mice. The dietary administration with CUSM at the highest dose (0.5%) significantly ($P<0.05$) reduced the number of BCAC in the +/+ (65% reduction) and db/db mice (74% reduction). CUSM at a dose of 0.1% also significantly lowered the number of BCAC in db/db mice (53% reduction, $P<0.05$). 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 BCAC when compared to their surrounding cryptal cells. Feeding with CUSM did not influence the stainability of Ob-R and IGF-1R. PCNA-labeling index was determined in BCAC that developed in the db/db mice (Groups 9–12). The mean PCNA-labeling indexes of Group 11 (AOM/0.1% CUSM) and Group 12 (AOM/0.5% CUSM) were significantly lower than that of Group 9 (AOM alone; $P<0.05$). The values of Groups 9 and 10 (AOM/0.02% CUSM) were comparable. A histopathological examination of the liver revealed the occurrence of fatty metamorphosis and fibrosis in the db/db mice that received AOM alone in contrast to the +/+ and db/+ mice. When the db/db mice were fed with 0.5% CUSM, these histopathological alterations were significantly inhibited fatty metamorphosis ($P<0.05$) and liver fibrosis ($P<0.05$).

All the measurements of total cholesterol, triglycerides, glucose, insulin, and leptin 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 the diet containing 0.5% CUSM. mRNA expression of the nonlesional colonic mucosa of Groups 2–4 were significantly lower than that of Group 1. iNOS expression of Groups 2–4 was also smaller than Group 1, but the differences were insignificant (Fig. 5).

**DISCUSSION**

The results of the Study 1 clearly indicated that 2 prenyloxycoumarins, auraptene and collinin, effectively inhibited AOM/DSS-induced, colitis-related colonic carcinogenesis without any adverse effects in mice. The suppressive effect of auraptene and collinin on the development of colonic adenocarcinoma was well correlated with the inhibition of cell proliferation activity, induction of apoptosis, and inhibition of immunoreactivity of COX-2 and iNOS in the colonic epithelial malignancies. These findings may suggest that dietary auraptene and collinin suppress IBD-associated colon carcinogenesis and are possibly applicable in human clinical trials.

The pathogenesis of IBD-associated colorectal carcinogenesis is widely believed to involve a step-wise progression from inflamed and hyperplastic cryptal cells through flat dysplasia to finally adenocarcinoma (79), but the mechanism is still unclear. However, mucosal inflammation may result in colonic carcinogenesis through several proposed mechanisms such as induction of genetic mutations, increased-cryptal cell proliferation, changes in crypt cell metabolism, bile acid enterohepatic circulation, and alterations in bacteria flora (80). These events are considered to promote IBD-associated CRC development. In the colon, the number of epithelial cells in the crypts is strictly regulated by a balance between cell proliferation and...
cell death that maintains homeostasis (81). In neoplastic tissues, changes in cell proliferation and apoptosis are regarded as a common denominator in the pathogenesis of tumor formation (82). It is thought that intermittent colonic epithelial damage and restitution caused by chronic inflammation contribute to the increased cancer risk in the long-term UC patients. The elevated rate of cell turnover associated with the epithelial damage-restitution cycle may increase the occurrence of mitotic aberrations and other genetic and epigenetic changes as well as take part in the promotion stage of cancer development (83). In this study, the modifying effects of auraptene and collinin on the cellular proliferation and apoptosis may contribute to their lowering activity in the incidence and multiplicity of colon adenocarcinomas.

Chronic inflammation is recognized as one of the major causes of human cancer (84). Inflammation-caused oxidative/nitrosative cellular damage is suspected to be responsible for the development of IBD-associated colorectal neoplasms. Therefore, certain antioxidants are effective as cancer chemopreventive agents. Auraptene suppresses 12-O-tetradecanoylphorbol-13-acetate-induced superoxide in human promyelocytic leukemia-60 cells; attenuates inflammatory leukocyte activation in vivo; and decreases inflammation, hydrogen dioxide production, and cell proliferation (85). In addition, auraptene likely reduces the production of lipid peroxidation products in rat colon carcinogenesis (42). These findings suggest that auraptene mitigates oxidative stress by suppressing oxygen radical generation by inflammatory leukocytes. Because nitrotyrosine production may involve in CRC development in this colitis-related mouse colon carcinogenesis model (43,59), our results suggesting potential use of the antioxidants collinin and auraptene in prevention of IBD-associated cancer may be caused by their suppression of oxidative/nitrosative cellular damage in our model. Given the correlation between increased COX-2 (86,87) or iNOS (88) expression and cancer occurrence in the inflamed colon of IBD patients, the chemopreventive effect of NSAIDs and iNOS inhibitors seems to be mediated, at least in part, by COX and iNOS inhibition (89). We (90) and others (91,92) have demonstrated that COX-2 inhibitors inhibited colon tumorigenesis as well as colitis induced by naturally occurring carcinogen. A specific inhibitor of iNOS is able to inhibit colon carcinogenesis in ApcMn/+ mice that received DSS (93). Suh et al. (94) synthesized novel synthetic triterpenoids that suppressed iNOS and COX-2 protein expression and demonstrated their potent differentiating, antiproliferating, and anti-inflammatory activities (95). Auraptene also can inhibit iNOS and COX-2 expression in RAW 264.7 cells treated with LPS and TNF-α (39). Our recent study (59) indicated that changes in inflammation scores paralleled with those of the nitrotyrosine immunohistochemical scores in the colonic mucosa, and these alterations in the inflamed colon resulted in powerful promotion effect of DSS in the AOM/DSS-induced mouse colon carcinogenesis. In this study, suppressing effects of dietary feeding with auraptene and collinin after the treatment with AOM and DSS might be mainly due to their inhibition of inflammation and oxidative/nitrosative stress in the colon.

The results of the Study 2 confirmed the high susceptibility of AOM-induced colon carcinogenesis in the obese/diabetic db/db mice in our previous findings (11). 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 obesity/diabetes and colon tumorigenesis. Our findings also suggest that insulin resistance involves CRC development (96). The main purpose of this study was to investigate the effect of CUSM on the early phase of AOM-induced colon carcinogenesis in the db/db mice. Because these lesions are considered to be putative precursor lesions of colonic adenocarcinoma (49), the results obtained clearly indicate the inhibitory effects of the dietary administration of CUSM on the development of AOM-induced ACF and BCAC in the db/db mice as well as the +/+ and db/+ mice. In this experiment, 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 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 to 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 (97). This association was also suspected by the findings in animal experiments (98) in which model animals for human familial adenomatous polyposis were used (76).

An association between diabetes and cancer was suggested over 100 years ago (99). The increased incidence of CRC in the Type 2 diabetic patients has been supported by a recent prospective, population-based cohort, case-control, and meta-analysis studies (54). Thus, there is an attractive hypothesis of insulin resistance CRC in which insulin resistant may thus be associated with the development of CRC (100), and this malignancy may therefore become a modifiable disease (101). 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 (57). Insulin and the IGF axis may be related to CRC development (102). 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 (103). We recently observed increased immunohistochemical expression of NF-κB (Fig. 6a) in the colonic neoplasms.
induced by the AOM/DSS treatment, suggesting that NF-κB is 1 of the good molecular targets of cancer chemoprevention (104) in the inflamed colon. In addition, certain citrus compounds, auraptene (22) and nobiletin (105), in diet decreased NF-κB-immunohistochemical expression in the malignancies (Figs. 6b and 6c; unpublished work). In this study, feeding with CUSM did not influence the serum level of insulin and immunoreactivities of IGF-1R and Ob-R in the BCAC in the db/db mice. However, the treatment reduced cell proliferation activity in the BCAC by estimating PCNA-labeling index. CUSM could reduce the occurrence or progression of BCAC through lowering the cell proliferation, although the exact mechanism(s) should be elucidated.

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 carcino genesis (106), thus increases the circulating leptin level (107). In addition, dietary fiber has been reported to decrease serum leptin concentration while reducing colon carcinogenesis by lowering the degree of cryptal cell proliferation (108). However, CUSM feeding did not affect serum leptin levels in the db/db mice. Thus, CUSM constituents other than fiber, that is, flavonoids, may have contributed to the reduction in the occurrence of putative precursor lesions, ACF and BCAC, in the colon of the db/db mice. Our recent study aimed to determine whether auraptene suppresses ACF and BCAC development in the db/db female mice initiated with AOM and indicated that dietary auraptene is able to inhibit the development of both precursor lesions (22). The suppression by dietary auraptene is considered to be caused by reducing serum triglycerides, inducing apoptosis, and/or reducing cell proliferation. Taken together, we suspected that hesperidin and auraptene in CUSM may be responsible for the inhibition of preneoplasia development in the db/db mice because this chemical can inhibit chemically induced colon carcinogenesis in rodents (109).

In conclusion, the results of Study 1 and Study 2 confirmed the high susceptibility of the inflamed mouse colon induced by DSS and obese/diabetic db/db mice to AOM-induced colon carcinogenesis. Our data provide further evidence that citrus compounds, including auraptene, collinin, and CUSM, are able to inhibit inflammation- and obesity-related colon carcinogenesis. Further studies focusing on the detailed mechanisms of inhibitory effects of citrus materials on the development of CRC in the inflamed colon and colon of obese mice should be carried out for the prevention and treatment of the colonic malignancies associated with these conditions.

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